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UNIVERSITY OF ALBERTA

PLATELET ACTIVATION BY COLLAGEN

BY

JIDONG LIAN ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

IN

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Date Nov 29. 93

To my parents, and my son.

For my loving wife, Yanning.
Her understanding, patience and
support were invaluable

ABSTRACT

Collagen is a major component of the blood vessel wall, and in fibrillar form is a potent platelet agonist, suggesting a role in platelet activation. The goal of this study was to characterize the mechanism of platelet activation by collagen. We investigated whether platelets use different mechanisms for platelet adhesion to monomeric collagen under dynamic versus static conditions. We also compared monomeric collagen to fibrillar collagen to assess the role of polymerization on collagen signaling.

Platelet adhesion was shown to require PLA2 activation under both dynamic and static conditions. PLA2 activity appeared to be regulated through the $\beta 1$ integrin subunit coupled to the Na^+/H^+ exchanger since platelet adhesion to collagen beads was dramatically inhibited by anti-$\alpha_2\beta_1$ or anti-β_1 integrin mAb, or by inhibitors of Na^+/H^+ exchange or PLA2 activation. Platelet adhesion mediated by force-dependent PLA2 activation appeared to require Ca^{2+} importation through a $\beta 3$ integrin-dependent process because platelet adhesion to collagen beads was completely prevented by removal of external Ca^{2+} with EGTA, or by an anti-$\beta 3$ integrin mAb. In contrast platelet adhesion to collagen under static conditions was greatly enhanced by both anti-$\beta 1$ and anti-$\beta 3$ integrin mAbs. mAb 145-89 directed against GPIV could block platelet adhesion under static condition, but was not effective under dynamic conditions. Monomeric collagen did not induce platelet aggregation in solution, but became an effective agonist following attachment to latex beads. The activation signal required thromboxane synthesis, an active Na^+/H^+ exchanger, and the involvement of the β_1 integrin subunit. In contrast platelet activation by fibrillar collagen did not require a Na^+/H^+ exchanger, or the involvement of the β_1 integrin subunit, and was less dependent upon thromboxane synthesis. These novel findings of significant differences in the mechanism of platelet activation under simulated *in-vivo* conditions as opposed to usual *in-vitro* conditions contribute to the better understanding of the mechanism of platelet- extracellular matrix interaction.

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LIST OF SYMBOLS AND ABBREVIATIONS

MAB:	MONOCLONAL ANTIBODIES
PLA2:	PHOSPHOLIPASE A2
PLC:	PHOSPHOLIPASE C
GP:	GLYCOPROTEIN
CD:	CLUSTER DESIGNATION
VWF:	VON WILLEBRAND FACTOR
KDA:	KILO-DALTON
ECM:	EXTRACELLULAR MATRIX
VLA:	VERY LATE ACTIVATION ANTIGEN
BSA:	BOVINE SERUM ALBUMIN
BPB:	4-BROMOPHENACYL BROMIDE
HMA:	5-(N,N-HEXAMETHYLENE) AMILORIDE
EIPA:	5-(N-ETHYL-N-ISOPROPYL) AMILORIDE
TXA2:	THROMBOXANE A2
PKC:	PROTEIN KINASE C
PAF:	PLATELET ACTIVATING FACTOR
PDGF:	PLATELET-DERIVED GROWTH FACTOR
NDGA:	NORDIHYDROGUAIARETIC ACID
AA:	ARACHIDONIC ACID
ADP:	ADENOSINE DIPHOSPHATE
BCECF:	[2',7'-BIS (CARBOXYETHYL-5 (AND 6)-CARBOXY) FLUORESCEIN
AM:	ACETOXYMETHYL
W7:	N-(6-AMINOHEXYL)-5-CHLORO-1-NAPHTHALENESULFONAMIDE

CHAPTER I: INTRODUCTION

A. BACKGROUND AND THESIS OBJECTIVE

The purpose of this study was to investigate platelet activation by immobilized monomeric collagen. The specific objectives of this thesis were:

1. To investigate the role of mechanical agitation in platelet adhesion by comparing adhesion to monomeric collagen immobilized on a plastic plates under static conditions with adhesion to collagen immobilized on latex beads under dynamic (stirring) conditions.
2. To compare aggregation induced by immobilized monomeric collagen with that induced by the known agonist polymeric (fibrillar) collagen.

Platelet activation by soluble agonists such as thrombin, ADP, or arachidonic acid occurs under stirring conditions *in vitro*, and is well studied. Activation results in platelet-platelet adhesion (aggregation) through the conversion of adhesion receptors to high affinity states, the rapid exocytosis of multimeric proteins which affect platelet-platelet adhesion by bridging receptors on opposing platelet surfaces, and the release of soluble agonists such as ADP and thromboxane A_2 which serve to recruit further platelets. Less well studied, but physiologically more relevant to primary hemostasis is the ability of platelets contacting immobilized extracellular matrix (ECM) proteins to adhere, and become activated. Defining the relative contribution of signals from soluble agonists, and insoluble agonists is a major unsolved problem in the understanding of the hemostatic response.

The ECM glycoproteins, including fibronectin, laminin, and various types of collagen, play a major role in regulating platelet activation (1, 2, 3, 4). The mechanisms whereby the ECM proteins regulate platelet function are under intensive investigation. It is clear that platelet membrane surface receptors, such as the integrins, are very important in the interaction of platelets with the ECM. Integrins are heterodimers consisting of noncovalently associated α and β subunits. They are clustered at sites of adhesion to the underlying ECM in regions known as focal adhesions which are thought to be important not only as structural links between the ECM and the cytoskeleton, but also as sites of signal transduction from the ECM. Interaction of cells with ECM are mainly mediated by the $\beta 1$ and $\beta 3$ integrin subfamilies. Previous studies have focused on characterizing the structures of integrins and their interaction with ECM proteins (5, 6). Although considerable work has been done to characterize platelet membrane receptors for ECM components, little is known about how the receptors transduce signals across the membrane to initiate platelet adhesion to the ECM. However, clues are beginning to emerge, suggesting that intracellular second messengers such as Ca^{2+}, cytoplasmic pH, and protein tyrosine phosphorylation may be critical for cellular activation by the ECM. Evidence that integrins modulate tyrosine kinase-mediated signal transduction was first obtained from studies of platelets. Agonist-induced tyrosine phosphorylation of the 125 kDa protein $pp125^{fak}$ is dramatically blocked when specific ligands are bound to GPIIb/IIIa, a major platelet integrin $\alpha IIb\beta 3$. In addition, platelets from patients who lack $\alpha IIb\beta 3$ fail to phosphorylate $pp125^{fak}$. Furthermore, an antibody against the $\beta 1$ integrin subunit also induces tyrosine phosphorylation of $pp125^{fak}$. These results suggest that integrins could be involved in signal transduction through tyrosine phosphorylation (6, 7). A number of other integrin-related signaling events have also been described. Platelet $\alpha IIb\beta 3$ integrin was shown to regulate Ca^{2+} flux across the plasma membrane (discussed later). Crosslinking of the $\beta 1$ integrins with mAb induces a transient elevation in intracellular pH (29, 30) suggesting that clustering of integrins may be sufficient for activation of Na^+/H^+ exchange. Since Ca^{2+} and H^+ transport is required for the activation of PLA2, both $\beta 1$ and $\beta 3$ integrins might be involved in the regulation of PLA2 activity. A recent report has confirmed the possibility of $\beta 1$ integrin-mediated activation of PLA2 (175). Activation of PLA2 and released arachidonic acid have also been shown to mediate spreading of HeLa cells on a collagen matrix (119). Thus, $\beta 1$ and $\beta 3$ integrin-mediated activation of PLA2 through transport of Ca^{2+} and H^+ may play an important role in regulating platelet adhesion to collagen. To gain insight into potential signaling pathways induced by platelet/ECM interactions we have focused our study on the effect of platelet adhesion to monomeric type I collagen.

Collagen is a major component of the blood vessel wall, and in its fibrillar form is a potent platelet agonist. Following damage to the vessel, platelets are brought into contact with the exposed subendothelium under conditions of hemodynamic flow - where they become activated by soluble factors and by vessel wall proteins (8, 9, 10, 11). Platelet activation appears to depend not only on the involvement of platelet membrane adhesion molecules, but also on the shear forces associated with blood flow (11, 12). Monomeric collagen molecules are not stimulatory in solution, but become so when they polymerize into fibrils (13, 14, 15, 16). Evidence that monomeric collagen can support divalent-cation-dependent platelet adhesion has been reported (17, 18); however, only adhesion to fibrillar collagen is reported to result in full platelet activation and secretion (17, 19). Collagen stimulated platelet activation has been studied *in vitro* by measuring platelet aggregation induced by suspensions of collagen fibrils in a platelet aggregometer, or by quantitating platelet binding to collagen matrices over 45 to 90 minutes, in most cases under static conditions. However, platelets in circulation do not encounter suspended fibrillar collagen, or attach to collagen surfaces in the absence of hemodynamic flow. The *in vitro* assay conditions therefore do not simulate the hemodynamic conditions to which platelets are exposed *in vivo*. In another model platelet adhesion to bead-coated collagen matrices under flow conditions was reported where beads were coated with fibrillar collagen, and agitated at low speeds (20). Adhesion in this model takes place slowly, and the platelets do not degranulate. Therefore, the existing experimental models either fail to distinguish between primary contact signals and subsequent platelet-platelet adhesion steps under flow (dynamic) conditions; or fail to activate platelets to the point of degranulation under static conditions. Since monomeric collagen can elicit primary activating signals, we hypothesized that platelets exposed to monomeric collagen immobilized on latex beads under stirring conditions might aggregate similarly to platelets exposed to a soluble agonist. Since the platelet aggregometer does not generate significant shear force, the signals generated should be independent of shear effects. Such a model would enable investigation of the sequence and nature of collagen contact signals. Since platelet adhesion to collagen in intravascular situations may require different mechanisms on the part of the platelet from adhesion in extravascular situations we hypothesized that different receptors and signals might be employed under dynamic and static conditions.

In this thesis we set out to re-examine the relationship between mechanical agitation and platelet activation with respect to collagen by exposing platelets to beads coated with monomeric collagen under stirring conditions. Our goal was to characterize the mechanism of platelet adhesion to immobilized collagen and to assess the role of mechanical agitation in collagen-induced contact signaling. The characteristics of platelet adhesion by mechanical agitation were compared to those of platelet adhesion by contact with plastic-immobilized monomeric collagen under static conditions in order to separate force-components from contact-components. We also wanted to find out if platelet aggregation induced by bead-immobilized monomeric collagen differed from that induced by fibrillar collagen in terms of the receptors involved and the signals generated. Since activation of platelets by collagen requires first that the platelets bind to it and secondly that the platelets degranulate, we divided our observations into the effect of mAbs and inhibitors upon adhesion and aggregation. To identify the receptors we observed the effects of receptor blockade using monoclonal antibodies (mAb). To identify the signals we employed pharmacological inhibitors.

B. PLATELET ACTIVATION

B-1. Different stages of platelet activation

Following the rupture of blood vessel wall, subendothelial components, such as collagen, are exposed to the circulating blood and come into contact with platelets. Blood platelets adhere to subendothelial elements through several sequential stages, namely platelet-subendothelium contact, followed by stimulation and spreading of these contact platelets. Contact platelets have the same feature as unactivated, discoid platelets in the circulating blood, but are firmly attached to the vascular surface and are not easily dislodged. Contact represents the initial event of platelet adhesion, the process by which an non-activated but moving platelet stops and sticks at a site of vascular damage. Once contact (adhesion) occurred, platelets become stimulated and spread along the surrounding subendothelial surface. Stimulation is a major aspect of platelet function, involving transmembrane signaling events, and cytoplasmic reorganization-pseudopod formation, accompanied by granule release (secretion). The released granule contents, such as ADP, TXA2 etc., amass additional platelets at the ruptured site and facilitate the platelet-platelet interactions called aggregation (31, 126). For convenience, we refer to the whole process as platelet activation.

B-2. Platelet activation by mechanical force

As blood moves along the vessel wall, platelets are forced laterally from the central blood stream towards the vascular surface where they encounter the subendothelial components at site of vascular damage. Shear force arises when two layers of material move at different rates or directions. At the interface between moving platelets and the blood vessel wall, shear forces are exerted on both the platelet and the vascular surface. This is a unique feature of platelet adhesion: the process depends on a shear force, one kind of mechanical force, which affects specific membrane molecules. The observation that under laminar flow platelet activation is proportional to shear forces existing between the moving platelet and the immobile ECM suggests shear force is an important determing factor. In an aggregometer, platelet activation is subjected to shear force due to stirring that is much less than that existing in the blood stream. The possible role of shear force cannot properly be evaluated using a platelet aggregometer. However, other physically determined events occur in the aggregometer when platelets are brought into contact with immobilized ECM which may be as, if not more, important than shear force. In this case platelet-ECM contacts are proportional to the stirring speed and may reflect a range of sensitivities to the frequency of contact stimulation.

It is reported from studies of platelet activation under conditions of hemodynamic flow that platelet activation will not occur unless sufficient shear forces are present (10, 11, 12, 21). Platelets bind immobilized vWf through activation of a shear-dependent receptor system. Shear-induced platelet aggregation involves large plasma vWf multimers and unusually large vWf forms derived from endothelial cells in addition to ADP, mobilization of intracellular Ca^{2+}, and membrane GPIb and GPIIb/IIIa (22, 23). Platelets respond to soluble vWf by the GPIb-dependent importation of Ca^{2+} through a mechanism potentiated by the interaction of vWf molecules with GPIIb/IIIa (24). Platelet adhesion to fibrillar collagen under conditions of shear force appears to be mediated by vWf (25). However, less well understood is how other mechanical forces are involved in regulating platelet-collagen interactions. Integrins are ECM receptors which may act as mechanochemical transducers to transmit mechanical signals into a biochemical response (26). As discussed above, members of the $\beta 1$ and $\beta 3$ integrin subfamilies when crosslinked are reported to induce protein tyrosine phosphorylation, mobilization of intracellular Ca^{2+}, and elevation of intracellular pH (6, 7, 27, 28). It has also been reported that ECM ligands have to both cluster integrins and prevent their internalization in order to sustain the activation of Na^{+}/H^{+} exchange (29, 30). Therefore, proteins immobilized on solid surfaces may induce different signals from the same protein in solution. These may involve not only substrate-induced changes in ligand conformation, but also the activation of mechano-responsive signaling pathways.

C. PLATELET MEMBRANE RECEPTORS

Platelet membrane proteins, glycoproteins in particular, act as receptors for a number of agonists that are capable of activating platelets. They are involved in the adhesion of platelets to subendothelial matrix and in platelet-platelet cohesion (aggregation). Many of these receptors have been identified, cloned, and sequenced. Most of these glycoprotein receptors are noncovalently linked heterodimers, including those that belong to the supergene family of adhesion receptors called the integrins which provide the linkage between ECM proteins and the cytoskeleton.

C-1. Platelet membrane glycoproteins

The major platelet membrane glycoproteins (Table1, 126) have been extensively studied. They can be divided into the integrin and non-integrin glycoproteins. The integrin family consists of $\alpha 2\beta 1$ (GPIa/IIa), $\alpha 5\beta 1$ (GPIc'/IIa), $\alpha 6\beta 1$ (GPIc/IIa), $\alpha v\beta 3$ (VnR), and $\alpha IIb\beta 3$ (GPIIb/IIIa). The non-integrin glycoproteins are: GPIb, GPIX, GPIV, GPV, and GPVI. These membrane glycoproteins serve as adhesion receptors, including a collagen receptor (GPIa/IIa; $\alpha 2\beta 1$), a fibrinogen receptor (GPIc'/IIa; $\alpha 5\beta 1$), a laminin receptor (GPIc/IIa; $\alpha 6\beta 1$), a promiscuous receptor (VnR, $\alpha v\beta 3$) for vitronectin, fibrinogen, vWf, and thrombospondin and a promiscuous receptor (GPIIb/IIIa; $\alpha IIb\beta 3$) for fibrinogen, fibronectin, vWf, vitronectin, thrombospondin, and collagen (32). GPIb/IX, a receptor for vWf, is the receptor responsible for platelet adhesion to the subendothelial matrix under hemodynamic conditions. GPIV is a receptor for collagen and possibly thrombospondin. GPV may be associated with GPIb/IX, but its function is unknown (31, 33). GPVI (P62) was recently reported by Moroi (34) to serve as a collagen receptor.

C-2. Other platelet proteins

The leukocyte differentiation antigen CD9, first described as a 24 kDa protein on pre-B cells, is a member of the tetra-span transmembrane supergene family (35), and is expressed on various cell types. CD9 is a major component of the platelet membrane with 45,000 copies per platelet (approximately equal to the number of GPIIb/IIIa receptors)(36). Anti-CD9 mAbs induce strong platelet aggregation and granule release, indicating that CD9 may play an important role in platelet aggregation (37). Anti-CD9 mAbs promote physical association between the CD9 antigen and the GPIIb/IIIa complex (38) suggesting a role in regulating the functional state of the integrin, and in transducing a signal from it.

CD63 antigen is a lysosomal membrane glycoprotein that has been identified as a platelet activation molecule with a molecular weight of 30-60 kDa (39, 40). This glycoprotein contains 238 amino acids with four putative hydrophobic transmembrane regions. CD63 has homology to CD9 and was shown to be identical to ME491, a protein originally described on the plasma membrane of malignant melanoma (41), and pltgp40, a 40 kDa platelet glycoprotein (42). The function of CD63 remains unknown.

The glycoprotein GMP-140 is the only member of the selectin gene family present in platelets and is a 140 kDa integral membrane glycoprotein present on the membrane of α granules. It becomes translocated to the surface of activated platelets upon granule secretion. GMP-140 functions as a receptor to mediate the adhesion of neutrophils and monocytes to activated platelets and to endothelial cells, and may also function as a recognition system for macrophages to remove activated platelets from the circulating blood (33).

D. BACKGROUND ON COLLAGEN

D-1. Historical overview

Collagen was first investigated by chemists involved in leather and gelatin production. The first model of collagen structure, described by Astbury in 1940, assumed that every third residue along the polypeptide chain was glycine and every ninth residue was hydroxyproline. Using electron microscopy, several researchers developed a model of the triple-helical structure of collagen in the early 1950s (43). Further chemical and biochemical investigation resulted in a detailed knowledge of collagen structure. Miller (44) discovered that the 70 nm-banded collagen fibrils observed in the electron microscopy studies were formed by three different, but homologous forms of collagen types known as types I, II, III. So far, 14 different types of collagen have been described. Those are described in detail elsewhere (43, 45, 46, 47, 48, 49).

D-2. Composition of collagen

Collagens are the most abundant proteins in mammals, accounting for about 30% of all proteins. Collagens are secreted mainly by connective tissue cells and assembled into collagenous fibers responsible for the functional integrity of tissues such as skin, bone, and cartilage. The collagen molecules contribute a structural framework to blood vessels and most organs. They are encoded by members of a superfamily of closely related genes. About 20 distinct collagen α chain subtypes have been identified, each encoded by a separate gene. Different combination of these genes are expressed in different tissues. Collagen monomers consist of three polypeptide α chains arranged in a triple helix which turns on a 3-residue Gly-X-Y sequence in which X and Y can be any amino acid with glycine as every third residue. Each chain is about 1,000 amino acid long. The amino acid composition is characterized by high proline, hydroxyproline, and glycine contents.

D-3. Distribution of collagens in the blood vessel wall

Platelet-collagen interaction results directly in platelet activation. Indeed, collagen appears to be among the most thrombogenic proteins yet identified in the blood vessel wall. Three of the 14 types of collagen readily form fibrils in the vessel wall including type I, which consists of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain; type II, which consists of three similar $\alpha 1(II)$ chains; type III, which consists of three identical $\alpha 1(III)$ chains. Two additional collagens are found in basal laminae and do not form fibrils: type IV, which consists of three $\alpha 1(IV)$ chains and type V, which consists of two $\alpha 1(V)$ chains and one $\alpha 2(V)$ chain. Type VI collagen, which consists of one $\alpha 1(VI)$ chain, one $\alpha 2(VI)$ chain, and one $\alpha 3(VI)$ chain, is present in all layers of large blood vessels and is widely distributed in connective tissues (43). Since type I and type III collagens represent about 80% to 90% of the total collagen in the blood vessel wall, they are likely to be the most important components in the activation of platelets by the severely damaged vessel wall. We have therefore restricted our study to the evaluation of type I collagen monomers.

D-4. Effects of collagen structures in platelet activation

Initial studies of the interaction between platelet and collagen were focused on the role of collagen in inducing platelet aggregation and secretion, and were not directed at the early events of platelet adhesion. A number of studies have shown that collagen-induced platelet aggregation and secretion had an absolute requirement for the native, triple helical structure of collagen (13, 16, 50). Monomeric collagen molecules have to polymerize into collagen fibers, or fibrils before they become a platelet agonist (13, 14, 15, 51). In order to determine whether monomeric collagen provided an effective substrate for platelet adhesion, Santoro (17), and Staatz (18) established the method of coating polystyrene dishes with different concentrations of monomeric, fibrillar, or denatured collagen. They found monomeric collagen could efficiently support Mg^{2+}-dependent platelet adhesion. They investigated the ability of several types of collagen to support platelet adhesion and found that the interstitial collagens, types I and III, and type IV collagen, major component of basement membrane, were effective substrates for platelet adhesion.

E. PLATELET RECEPTORS FOR COLLAGEN

A number of platelet membrane proteins have been proposed as potential mediators of platelet adhesion to collagen and mediators of collagen induced platelet aggregation (5, 31, 33, 52, 53, 126). Among them $\alpha 2\beta 1$ (GPIa/IIa), and GPIV (CD36) are the best-defined collagen receptors.

E-1. GPIa/IIa ($\alpha 2\beta 1$, VLA-2)

A well-known platelet collagen receptor GPIa/IIa is identical to the very late activation antigen-2 (VLA-2) complex, a member of the β_1 integrin subfamily, and can mediate the Mg^{2+}-dependent adhesion of platelets to collagen types I, II, III, and IV. Strong indirect support for GPIa as a platelet collagen receptor was reported by Nieuwenhuis (87) who described a patient with a mild bleeding disorder attributable to a defect in platelet GPIa. Platelet-functional studies revealed abnormal platelet adhesion to collagen, and impaired collagen-induced aggregation, but normal response to all other agonists. This was the first evidence that GPIa plays an important role in the platelet-collagen interaction. In order to verify the platelet membrane component which mediates the platelet adhesion to collagen, Santoro (17) developed a procedure for studying the adhesion of activated platelets to various substrates in the presence of divalent cations. When experiments were conducted in the presence of Mg^{2+}, a single polypeptide of 160 kDa was found. In a subsequent study, Santoro (88) showed that a heterodimeric complex of two polypeptides of 160 kDa and 130 kDa comprised the collagen-binding protein. Pischel (89) reported that the 160 and 130 kDa complex was specifically immunoprecipitated by mAb 12F1 (anti-GPIa/IIa mAb). This result confirmed that the GPIa/IIa complex, first isolated by Santoro, was the collagen-binding protein. Kunicki (90) also obtained evidence that the platelet membrane GPIa/IIa complex mediated adhesion of platelets to collagen. The mAb P1H5 (anti-GPIa/IIa mAb), which inhibited the platelet adhesion to collagen, also specifically immunoprecipitated a platelet surface glycoprotein corresponding to GPIa/IIa. This independent work complemented the conclusion regarding the platelet surface collagen binding-protein observed by Santoro. In a subsequent investigation, Staatz (91) found that adhesion of platelets to collagen via GPIa/IIa was Mg^{2+}-dependent and inhibited by Ca^{2+} in both platelets and liposomes containing the purified GPIa/IIa complex. These results suggested that separate binding sites for Mg^{2+} and Ca^{2+} might stablize different divalent cation-dependent structures within the GPIa/IIa complex. In a following study (18), Staatz carried out proteolytic digestion of this protein in the presence of Mg^{2+}, Ca^{2+}, Mg^{2+} and Ca^{2+}, or EDTA and mapped the digested peptides by SDS-

PAGE using both one and two-dimentional techniques. The results suggest that Mg2+ and Ca2+ stabilize different structures within the GPIa/IIa complex and that these structures influence both the collagen-binding activity and proteolytic susceptibility of the complex. As mentioned above, Coller (20) indicated that collagen-platelet interaction was regulated directly via GPIa/IIa, and indirectly via GPIIb/IIIa. Parmentier (92) reported that mAb LYP22, the first mAb directed against GPIIa (β1 integrin subunit), could inhibit platelet adhesion to type III collagen. In the presence of mAb LYP22, collagen-induced platelet aggregation was significantly prolonged, strongly suggesting that the mAb LYP22 might be involved in the initial platelet-collagen interaction. In Moroi's study (19) of platelet-collagen interaction, the first phase reaction depended on GPIa/IIa, confirming that GPIa/IIa was involved in the initial platelet-collagen interaction. Recently, Kainoh (93) provided the first evidence that ADP-induced platelet adhesion to collagen was almost completely blocked by anti-GPIa/IIa and anti-GPIIa mAbs suggesting that platelet adhesion to collagen induced by ADP was also mediated by the GPIa/IIa complex. $\alpha 2\beta 1$ integrin (GPIa/IIa, VLA-2) is not only expressed on the platelet surface, but also on other cell surfaces, such as fibroblasts, endothelium, epithelium, melanoma cells, and leukocytes (53, 94, 95, 96). Therefore, GPIa/IIa is a well-defined collagen receptor which plays a significant role in regulating platelet and other cell functions.

E-2. GPIV (CD36)

GPIV, an 88 kDa platelet membrane glycoprotein is reported to play an important role in platelet interaction with collagen and thrombospondin (73, 74, 75, 76, 77, 78). Purified GPIV in solution competed with platelet membrane-bound GPIV for added collagen and caused a progressive decrease in both aggregation and secretion responses. However, GPIV did not affect platelet aggregation induced by thrombin, ADP, epinephrine, arachidonic acid, and ionophore A23187. Fab fragments derived from a polyclonal antiserum specific for GPIV inhibited collagen-induced platelet adhesion and aggregation (74). Tandon (75) also showed that blood platelets of the Nak^a-negative phenotype did not express GPIV and were deficient in the initial stage of adhesion to fibrillar collagen, especially under Mg^{2+}-free conditions. These results suggested an important role for GPIV in the platelet-collagen interaction but left unanswered questions about the ability of GPIV to serve as a thrombospondin receptor (76, 78, 79, 80).

E-3. GPIIb/IIIa (αIIbβ3)

The GPIIb/IIIa complex is composed of one 140 kDa molecule of GPIIb and one 150 kDa molecule of GPIIIa, noncovalently associated on the platelet surface (81, 82). On unstimulated platelets, GPIIb/IIIa is randomly dispersed on the platelet surface and can only recognize immobilized fibrinogen. On activated platelets, GPIIb/IIIa complex forms patches on the platelet surface and functions as a promiscuous receptor for several soluble adhesive proteins, including fibrinogen, fibronectin, vWf, vitronectin, thrombospondin (32, 82), and collagen (19, 20, 83, 84, 85, 86). The ability of a monoclonal antibody PMI-1 directed against platelet GPIIb to inhibit the Mg^{2+}-dependent adhesion of platelets to collagen was reported by Shadle (83, 84). Fab fragments derived from the antibody were also shown to inhibit platelet adhesion to collagen. The inhibition could be blocked by the purified antigen. However, the purified antigen only partially inhibited the ability of a polyclonal antiserum to inhibit platelet adhesion to collagen. This result indicated that if the GPIIb/IIIa complex was directly involved in a primary mechanism of platelet adhesion to collagen it was not the only component involved in this process. Kotite (85) reported that GPIIb/IIIa complex could specifically cross-link to collagen when the surface-labeled platelets were allowed to attach to collagen and then treated with a chemical crosslinking agent. Tsunehisa (86) also found that GPIIb was the most abundant fraction of platelet surface protein bound to collagen. In a collagen-platelet interaction study, Coller (20) showed that anti-GPIIb/IIIa mAb had a minor inhibitory effect on platelet adhesion to collagen or collagen-induced platelet aggregation in either the absence or presence of plasma indicating that collagen could indirectly interact with GPIIb/IIIa and platelet-collagen interaction might take place in part through the GPIIb/IIIa complex. Coller suggested that the early interaction between platelets and collagen in the presence of plasma proteins was mediated primarily via GPIa/IIa, and that later events involve both receptors. Recently, Moroi (19) has suggested that the first reaction of platelet adhesion is a platelet-collagen interaction which is dependent on GPIa/IIa, and the second reaction is a platelet-platelet interaction which is dependent on GPIIb/IIIa. Overall, although GPIIb/IIIa can act as a receptor for collagen, it may only be involved in the late stages of platelet-collagen interaction.

E-4. VWf, fibronectin, and thrombospondin

VWf , which serves as an adhesive ligand between platelets and vascular components contains distinct binding domains including those for collagen, GPIb, and GPIIb/IIIa. VWf can mediate platelet adhesion to collagen through GPIb and GPIIb/IIIa under conditions of hemodynamic flow (10, 11, 24, 54, 55, 56, 57, 58), but can bind to platelets only when it is activated (59). Fibronectin, a plasma, cell surface and ECM glycoprotein, is composed of functional domains capable of binding to cell surface receptors as well as to other ECM molecules, such as collagen. It functions not in the earlier recognition of collagen by platelets, but in late events of platelet adhesion to collagen and the process of platelet aggregation (60). Fibronectin is localized in the α granules of platelets and is secreted in response to agonists such as collagen or thrombin (61). Thrombospondin, a high molecular weight glycoprotein first isolated from the supernatant of thrombin-stimulated platelets (62) is also a component of platelet α granules, and can also bind collagen, but is only secreted from α granules on platelet activation (63). Therefore, these three proteins may be involved only in late events in platelet activation, and may not act as collagen receptors.

E-5. 65 kDa, 61 kDa, 62 kDa, and 80 kDa proteins

A 65 kDa protein was described as a receptor for collagen by Chiang (64), according to the fact that the α1 chain of chicken skin collagen which recognized a 65 kDa protein and the α1CD5 peptide derived from it could induce platelet activation. These results were in contrast to many other observations that only native triple helical collagen was an effective agonist of platelet aggregation. Presumably, this exclusive feature of the chick skin α1 collagen chain and its peptide belong to an as yet uncharacterized property of the primary structure. Chiang (65) also showed that a polyclonal antiserum specific for the 65 kDa protein inhibited collagen-induced platelet aggregation. A 61 kDa glycoprotein, identified by Kotite (66), could bind to insoluble collagen. A 44 kDa proteolytic fragment of the 61 kDa polypeptide retained collagen binding activity. Although the molecular weight of this glycoprotein was similar to the 65 kDa protein, these two proteins were not the same polypeptide. A 62 kDa protein (P62), defined as platelet membrane GPVI (34), was suggested as a putative collagen receptor by Moroi (19, 34), Sugiyama (67), and Ryo (68). They reported that an antibody which recognized P62 could inhibit collagen-induced platelet aggregation and a patient who lacked P62 was unresponsive to collagen. They indicated that P62 played a crucial role as a collagen receptor in platelet activation. These reports leave significant questions about the relationship among P62, the 65 kDa protein, and the 61 kDa protein. A platelet surface 80 kDa protein may also represent a mediator of platelet adhesion to collagen (69). Surface-labeled platelets were attached to a column containing fibrillar collagen.

Following treatment with SDS some surface labeled proteins which still remained associated with the column were the polypeptides of 80 kDa protein, suggesting that the 80 kDa protein might represent a physiologically relevant platelet surface collagen binding protein.

E-6. Glucosyltransferase and transglutaminase

A platelet surface collagen glucosyltransferase was suggested to mediate platelet adhesion to collagen. Jamieson (70) proposed that the formation of an enzyme-substrate complex between the incomplete carbohydrate chains of collagen and the platelet surface enzyme was the means by which platelets adhered to collagen. However, Menashi (71) showed that only denatured collagen was a substrate for the glucosyltransferase, whereas native collagen was required to initiate platelet aggregation. Platelet factor XIII, a transglutaminase, was described by Saito (72). They pointed out that a zymogen form of platelet factor XIII was located on the surface of platelets and might play a role as a receptor for collagen. An antibody against plasma factor XIII was shown to induce platelet aggregation. Although glucosyltransferase and platelet factor XIII could mediate platelet activation by collagen, they may not directly act as collagen receptors.

F. PLATELET ACTIVATING SIGNALS INDUCED BY COLLAGEN

F-1. Overview of platelet signal transduction

Activating and inhibitory pathways of signal transduction in platelets are initiated by occupancy of platelet surface receptors for specific extracellular ligands. The receptor in turn interacts with one or more coupling proteins in the membrane which are known as guanine nucleotide-binding regulatory proteins (G proteins). G proteins interact with target enzymes, or ion channels in the plasma membrane, generating second messengers and modulating ion fluxes. Second messengers in turn stimulate protein kinases that phosphorylate intracellular enzymes regulating key aspects of platelet responsiveness (97).

F-2. Two major activating pathways involved in collagen induced platelet activation

The exposed collagen fibers in the ruptured blood vessel wall are the most physiological substrate for platelet adhesion which is dependent on the platelet membrane receptors as noted above. Following the predominant event of platelet adhesion to collagen, the initial responses are the activation of common biochemical pathways within the platelet, including the activation of PLC and PLA2 pathways (98, 99, 100, 101, 102). The stimulation of these pathways results in platelet release of constituents from intracellular granules, such as the aggregating agents ADP and thromboxane A2 (TXA2).

Collagen-mediated activation of PLC leads to the generation of two second messengers (Fig.18): DAG (diacylglycerol) and IP3 (inositol 1,4,5-trisphosphate) (103). DAG is considered to be important for activation of protein kinase C (PKC) which phosphorylate a 43 kDa protein P43, and is also implicated in two main metabolic routes: it is phosphorylated by the enzyme DAG kinase to form phosphatidic acid (PA), and it can produce arachidonic acid through a DAG lipase, therefore involving TXB2 formation (97, 104). IP3 mobilizes release of intracellular Ca^{2+} which in turn activates PLA2 and the calmodulin-dependent myosin light chain kinase to phosphorylate P20 (97, 105, 106). The mechanism of PLC signaling pathway stimulated by collagen requires thromboxane synthesis (101, 107), actin polymerization (101), Na^{+}/H^{+} exchange (108), and GTP binding proteins (109).

Collagen-induced platelet activation is also due to the stimulation of PLA2 which in turn cleaves membrane phopholipids producing arachidonic acid and lysophospholipids. The released arachidonic acid is converted to thromboxane A2 (TXA2) which activates PLC through occupation of a G-protein-coupled receptor (110, 111). In association with collagen, TXA2 induces the further activation of phospholipases (100, 107). Several mechanisms have been proposed to be involved in the regulation of PLA2 activity in platelets. PLA2 activity may be inhibited by the endogenous protein termed lipocortin, which has been shown to be blocked through PKC-mediated phosphorylation (113). However, lipocortin binds to the phospholipid substrate and not to the enzyme, and therefore does not inhibit PLA2 as long as the substrate is not limiting (114). Activation of cytosolic PLA2 (cPLA2) is dependent on elevation of intracellular Ca^{2+} which comes from either PLC-mediated IP3 pathway (97, 105, 106, 144) or importation of Ca^{2+} across the plasma membrane through Ca^{2+} channel (112, 117). The increased intracellular Ca^{2+} results in activation of PLA2 through translocation of cPLA2 from the cytosol to the membrane (112, 123, 124). cPLA2 may also be activated by local pH which is mediated by Na^+/H^+ exchange (116). In addition, G-protein was reported to regulate PLA2 activation (115, 125). Phosphorylation-mediated activation of cPLA2 appears to act synergistically with Ca^{2+} to fully activate cPLA2 (123).

The regulation of PLA2 and PLC appears closely interrelated, but they are independently controlled and are separable events (122). Although the relative importance of these pathways remains to be determined, PLA2 action was shown to be an initial event in collagen-mediated platelet activation and TXA2 was necessary to induce PLC action and further activation of PLA2 (107, 118, 120, 121). The PLA2 pathway was recently shown to be involved in regulating the interaction of HeLa cells to collagen since inhibition of PLA2 blocked cell spreading, and addition of exogeneous arachidonic acid overcame this inhibitory effect. In addition, inhibition of PKC did not block cell spreading, indicating that PKC activation was not essential for spreading (119). Therefore, collagen-induced platelet secretion and aggregation is absolutely dependent on the release of arachidonic acid (101).

F-3. Na^+/H^+ exchange in mediating platelet activation

Na^+/H^+ exchange plays an important role in regulating platelet activation. It is an electroneutral process controlled by the concentrations of Na^+ and H^+ on either side of the membrane. Removal of extracellular Na^+ or reduction of extracellular pH could block platelet aggregation and secretion (127, 128). Na^+/H^+ exchange is also blocked by amiloride or other amiloride analogues, such as HMA and EIPA. The most direct studies of intracellular pH in platelets were performed by Horne (129) who showed that amiloride blocked low dose thrombin-induced platelet activation, but not activation by high concentrations of thrombin. Later, platelet aggregation and secretion in response to a number of "weak agonists", such as epinephrine, ADP, PAF, A23187, and ionomycin were shown to involve Na^+/H^+ exchange (108, 130). In contrast to the "weak agonists", collagen failed to stimulate detectable changes in intracellular pH (131). It is possible that the pH change is too small to measure although Na^+/H^+ exchange is active. Activation of Na^+/H^+ exchange results in a rapid elevation in intracellular pH. This alkalinization may play an important role in stimulating PLA2 activity. The activated Na^+/H^+ exchanger enables PLA2 to function at low concentrations of intracellular Ca^{2+} (116, 130). The interdependent regulation of PLA2 by pH and Ca^{2+} may be especially important for "weak agonists" because these agonists produce modest changes in Ca^{2+} concentration and cytosolic pH. It has been suggested that Na^+/H^+ exchange may play a major role in signal transduction from the ECM (6, 7). Cell adhesion to the ECM, or antibodies against integrins can stimulate Na^+/H^+ exchange and elevate intracellular pH (pHi) (29, 137, 138, 139). Crosslinking of integrins with soluble antibodies induces a transient elevation of pHi that is independent of cell shape (30), suggesting that integrins can act independently of the cytoskeleton to regulate a cytoplasmic second messenger. Inhibition of Na^+/H^+ exchanger activity substantially blocks DNA synthesis (136, 140) indicating that Na^+/H^+ exchanger activity may mediate cellular proliferation.

F-4. Ca^{2+} in mediating platelet activation

Following platelet activation, the cytoplasmic Ca^{2+} concentration is increased. The increased intracellular Ca^{2+} which is involved in all of stages of platelet activation including shape change, aggregation, and secretion (141) comes from internal stores, such as the dense tubular system, and from Ca^{2+} influx across the platelet plasma membrane. The rapid rise in free calcium from the basal level of 0.1 µM may reach up to 1 millimolar concentration following platelet activation by thrombin, ADP, PAF, arachidonic acid etc.(142) whereas collagen only causes a low level elevation of intracellular Ca^{2+} (143). Platelet adhesion to collagen appears to be dependent on Mg^{2+} and inhibited by external Ca^{2+} (17). Smith (143) confirmed this observation, and further indicated that platelet adhesion to collagen was accompanied by a small increase in cytosolic free Ca^{2+}, even at high concentrations of collagen. This increase in cytosolic Ca^{2+} required the expedition of metabolic energy and actin polymerization (145) suggesting a requirement for cytoskeletal assembly. Recently, Smith (146) showed that a collagen-induced increase in intracellular Ca^{2+} was required for granule release and arachidonic acid liberation. Therefore, elevation of intracellular Ca^{2+} may be very important in collagen-mediated platelet activation.

Ca^{2+} influx across the plasma membrane is largely mediated by calcium channels, but the mechanisms that regulate Ca^{2+} influx are mostly unknown. The platelet membrane GPIIb/IIIa complex, a major platelet integrin, has been implicated in the regulation of Ca^{2+} influx across the plasma membrane of resting platelets (117, 147). The maximum rate of Ca^{2+} exchange in thrombasthenic platelets which lack GPIIb/IIIa complex is half the rate in normal platelets, suggesting that the GPIIb/IIIa complex may either be a calcium channel protein or be associated with one. Powling (148) provided evidence that the GPIIb/IIIa complex was not a Ca^{2+} channel protein, but that Ca^{2+} influx was mediated through a channel adjacent to the GPIIb/IIIa complex. Similarly, Hardisty (149) demonstrated that Ca^{2+} influx in response to ADP was significantly inhibited by antibodies to the GPIIb/IIIa complex which also inhibited fibrinogen binding and aggregation. Ca^{2+} influx induced by ADP on thrombasthenic platelets was normal and not inhibited by a monoclonal antibody to GPIIb/IIIa indicating that ADP involves Ca^{2+} entry through other molecules. Yamaguchi (150) reported that Ca^{2+} influx in response to thrombin and collagen was partially blocked by a monoclonal antibody against the GPIIb/IIIa complex. Rybak (151, 152) indicated that purified GPIIb/IIIa complex could facilitate Ca^{2+} movement across a phospholipid layer. A monoclonal antibody P256, which is specific for GPIIb/IIIa complex, could directly induce calcium mobilization (153). Recently, Ellen (154) found that calcium channel blockers significantly inhibited calcium influx into GPIIb/IIIa liposomes further indicating the involvement of GPIIb/IIIa in regulating Ca^{2+} movement. In conclusion, GPIIb/IIIa may play an important role in regulating Ca^{2+} flux across the plasma membrane.

A human leukocyte differentiation antigen CD9 is also expressed on the platelet surface membrane (37). The widespread tissue distribution of the CD9 antigen could indicate a basic biological function perhaps relating to calcium flux. Stimulation of CD9 antigen resulted in calcium uptake into isolated membrane vesicles (155, 156). Several monoclonal antibodies against CD9 were shown to elevate intracellular calcium concentration, and calcium influx from the extracellular media appeared to contribute significantly to intracellular calcium elevation (157, 158, 159). Since CD9 may contribute to an increase in intracellular calcium, and may physically associate with GPIIb/IIIa complex (38), it may play a role in regulating GPIIb/IIIa-dependent calcium fluxes.

F-5. Tyrosine phosphorylation in mediating platelet activation

There are two concepts on the mechanism of integrin-mediated signaling. One view is that integrins mediate signals by reorganizing the cytoskeleton, thus regulating cell shape and internal cellular structure. An alternative mechanism is that integrins are deemed to be true receptor capable of generating biochemical signals within the cell. Several reports have suggested that ligation of integrins can change cellular patterns of tyrosine phosphorylation (6,7). Evidence that integrins regulate tyrosine kinase-mediated signal transduction was first obtained from studies of platelets. Agonists that trigger platelet aggregation can induce tyrosine phosphorylation which is profoundly altered when specific ligands are bound to platelet membrane integrin $\alpha IIb\beta 3$ (176). More definitive evidence has been obtained that an antibody against the $\beta 1$ integrin subunit also induces tyrosine phosphorylation (177). These results suggest that local crosslinking of integrins is sufficient to trigger activation of tyrosine kinase activity. Recently, a strong correlation has been observed between ligation of platelet integrins and activation of the 125 kDa protein termed "pp125 Focal Adhesion Kinase" ($pp125^{fak}$) (178); for example, stimulation of $pp125^{fak}$ is dramatically reduced in $\alpha IIb\beta 3$ deficient platelets (178). Thus, ligation of integrins followed by integrin clustering and reorganization of cytoskeleton can affect $pp125^{fak}$, a novel cytoplasmic tyrosine kinase, leading to activation and increasing tyrosine phosphorylation of this protein. Therefore, it seems likely that integrin-mediated activation of $pp125^{fak}$ is an early step in a signal transduction cascade that allows the flow of information from the extracellular matrix to the cell interior.

CHAPTER II: MATERIALS AND METHODS

A. DRUGS AND BIOLOGICALS

A-1. Monoclonal antibodies (mAb)

MAb AP1, mAb P2, and mAb SZ21 are directed against glycoprotein (GP) Ib, GPIIb/IIIa, and GPIIIa respectively and were obtained from AMAC, Westbrook, ME. MAb JBS-2, an anti-GPIa/IIa mAb, was the kind gift of Dr. John Wilkins (Department of Medicine, University of Manitoba). MAb 145-89, and mAb 145-70, are both IgG2b mAbs, produced by Dr. A.R.E. Shaw's lab (Deparment of Medicine, University of Alberta and Cross Cancer Institute), and are directed against GPIV and CD63 respectively. MAb 4B4, an anti-β_1 integrin mAb, was obtained from Coulter, Hialeah, FL. MAb ESvWf7, an anti-vWf mAb was purchased from American Diagnostic, Biopool, Ont.

A-2. Reagents

Apyrase (Grade V), prostaglandin I_2, fibrinogen, bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), adenosine diphosphate (ADP), thrombin (bovine), arachidonic acid (AA), ethylenediaminetetracetic acid (EDTA), ethyleneglycol-bis (b-aminoethyl ether)-N, N'-tetracetic acid (EGTA), cytochalasin B, staurosporine, sphingosine, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), indomethacin, neomycin,

nordihydroguaiaretic acid (NDGA) were purchased from Sigma, St. Louis, MO. 4-bromophenacyl bromide (BPB) was obtained from Aldrich Chem. Inc, Milw.,WI. Genistein was obtained from UBI, Lake Placid, NY. Phospholipase A2 (PLA2) (from bee venom) was purchased from Boehringer, Mannheim, Germany. The thromboxane receptor inhibitor BM13.177 was the kind gift of Dr. Stegmeier, Boehringer, Mannheim, Germany. 5-(N,N-hexamethylene) amiloride (HMA), and 5-(N-ethyl-N-isopropyl) amiloride (EIPA), the Na^+/H^+ exchanger blockers, were obtained from Research Biochemicals Inc., Natick, MA. Collagen Vitrogen 100 was used for immobilized collagen study and was purchased from Celtrix Laboratories, California. Fibrillar type I collagen reagent for control study was purchased from Helena Laboratories, Beaumont, Texas. Different sizes of polystyrene latex beads were purchased from Sigma, St. Louis, MO, or from Polysciences, Inc., Warrington, PA. BCECF [2',7'-bis (carboxyethyl-5 (and 6)-carboxy) fluorescein], an ion-sensitive dye for measuring intracellular pH, was obtained from Molecular Probes, Inc., Eugene, OR. All other reagents used in this study were obtained from Sigma, St. Louis, MO. All pharmacological inhibitors were prepared as stock solutions (in buffer, DMSO, ethanol, or chloroform) depending on the reagents being investigated.

B. MEDIA

B-1. Tyrode's/Hepes platelet buffer

Sodium Tyrode's/Hepes platelet buffer, pH 7.2, containing 140 mM NaCl, 0.36 mM NaH_2PO_4, 14.7 mM Hepes, 1% BSA (w/v), and 3.5 mg/ml glucose, was used for washing the platelets. For investigation of the effect of Na^+ concentration, platelets were resuspended in Na^+ Tyrode's/Hepes platelet buffer, or in the buffers of identical composition except that NaCl was replaced by an equal concentration of KCl or choline chloride.

B-2. Tyrode's/Hepes bead buffer

For washing polystyrene latex beads to coat with collagen, bead buffer, pH 3.5, containing 140 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 14.7 mM Hepes, 3.5 mg/ml dextrose, was used.

B-3. NP-40 platelet lysis buffer

This buffer, containing 20 mM Tris.HCl, 1 mM Na_2HPO_4, 0.137 M NaCl, 0.5% (v/v) NP-40, and 10 mM iodoacetamide, pH 8.0, was used to lyse platelets determining platelet adhesion to collagen matrix under static conditions with BCECF AM.

C. PREPARATION OF WASHED PLATELETS

Human platelets were collected from volunteers who denied taking any medication for the previous two weeks. Blood was drawn into acid/citrate/dextrose vacutainer tubes (Becton-Dickinson, Mississauga, Ont.). Platelet rich plasma (PRP) was collected by centrifugation of the whole blood at 150 xg for 10 minutes at room temperature. The PRP was made 10 ng/ml in prostaglandin I_2, and 1 unit/ml in apyrase (Grade V) to prevent platelet activation during the washing procedure. Washed platelets were collected by centrifugation at 550 xg for 10 minutes. The platelet pellet was resuspended in Tyrode's/Hepes platelet buffer and their concentration was adjusted to 2 x 10^8 platelets/ml. The washed platelets remained at room temperature (20-22^{o}C) for the duration of the test. The assay was performed within 2 hours.

D. PREPARATION OF COLLAGEN-COATED LATEX BEADS

D-1. Separation of monomeric collagen

3 ml of Vitrogen 100 collagen filtered by passing through a 300 kDa filter (Sin-Can Inc., Calgary, AB) was centrifuged with a fixed angle rotor and 17 mm diameter tube adaptors at 5000 rpm for 60 minutes. Monomeric collagen, which is below 300 kDa in size, passed through the filter, whereas polymeric collagen assemblies were retained. About 1 ml of monomeric collagen could be obtained at each centrifugation due to plugging of the filter.

D-2. Procedure for making collagen-coated beads

15 to 200 µl of different sizes of polystyrene latex beads (Sigma, or Polysciences) were washed three times with Tyrode's/Hepes bead buffer and incubated with 0.2 ml solution of monomeric collagen

(1mg/ml) for 1 hour at room temperature with constant rotation. The beads were pelleted, resuspended in 100 μl of 5% bovine serum albumin (BSA) for 1 hour at room temperature on a rotary shaker, and washed once in Tyrode's/Hepes bead buffer. Control beads were treated with BSA, and washed three times. The beads were resuspended in a final volume of 100 μl of Tyrode's/Hepes bead buffer. During this procedure, pH was kept at pH 3.5 to avoid polymerization of monomeric collagen at high pH. The beads could be used within one week or even longer.

D-3. Determining the volumes of the beads used

For comparison of the ability of different sized beads coated with collagen to induce platelet aggregation, same surface areas of different sized beads were chosen. The volumes of different sized beads were determined by the following equations:

$$\text{Surface area of a bead} = \pi d^2 \tag{1}$$

$$\text{Volume of a bead} = \pi d^3/6 \tag{2}$$

$$V_T = 100\,\pi\, d_1{}^2\, d_2\, N_1/6X = 52.36\, d_1{}^2 d_2 N_1/X \tag{3}$$

In equation (3), V_T is the total volume of bead$_2$ needed, d_1 is the bead$_1$ diameter, d_2 is the bead$_2$ diameter, N_1 is the known number of bead$_1$, and X is the percentage of solid bead in its solution.

D-4. Calculation of collagen fixed on the beads

A standard curve for different concentrations of collagen was established by UV spectroscopy. Nominal concentration of collagen fixation by the beads was determined by the concentration difference between the whole collagen solution and the collagen remaining in the solution after the beads were pelleted. For each loading, the concentration of loaded collagen on the beads was 0.13 ± 0.02 mg/ml in solution.

E. AGGREGOMETRY AND DETERMINATION OF PERCENT AGGREGATION

Platelet aggregation in washed platelets at 2 x 10^8 platelets/ml was performed at 37°C with constant stirring at 900 rpm using a Payton Aggregometer (Payton, Scarborough, Ont.). Percent aggregation was determined by setting the baseline of light transmission with washed platelets to 10, and the maximal light transmission with Tyrode's/Hepes platelet buffer to 90 on the chart recorder. This allowed the percent aggregation to be calculated from the final chart reading (CR) by the following equation:

$$\text{Percent Aggregation} = (\text{CR-10/90-10}) \times 100 = (\text{CR-10/80}) \times 100 \quad (4)$$

All aggregation assays were performed in the presence of 0.2 mg/ml of fibrinogen except where mentioned. Platelets (300 µl at 2 x 10^8 platelets/ml) were placed in aggregometer tubes. Either 2 mM Ca^{2+} or 2 mM Mg^{2+} was added to each tube according to the experiments.

The concentration of the agonists and inhibitors were chosen empirically by obtaining minimal concentration required to produce normal aggregation or inhibition, and by using recommended concentrations from the experimental literature. Inhibitors or mAbs were normally preincubated with platelet suspensions in the tubes for 3 minutes at 37°C with stirring prior to the addition of agonists. Since the immobilized beads were opaque, the aggregometer reading was adjusted to compensate for the reduction in light transmission on addition of the beads. The aggregometer figures shown were representative of the results obtained from a minimum of three different donors. The concentration of bead-bound collagen used in each of experiments was 0.4 μg/ml and was the minimal concentration required to induce maximal platelet aggregation.

F. PLATELET ADHESION TO MONOMERIC COLLAGEN UNDER STATIC CONDITIONS

The assay used in this study for measuring intracellular pH was BCECF AM [2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxy-methyl ester], which has become the most widely used intracellular fluorescent pH indicator. This dye is typically used as a dual excitation indicator. The ratio of excitation intensity at 490 nm relative to 440 nm is pH sensitive. BCECF is calibrated in situ with 10 μM concentration of the ionophore nigericin in the presence of high potassium (140 mM) to eliminate potential gradients across cellular membranes. By performing careful calibration at the end of each experiment, it is possible to use BCECF to obtain measurement of pH_i

from about pH 6.5-7.5. The procedure for platelet adhesion to monomeric collagen using this assay was as follows:

F-1. Preparation of BCECF AM loaded platelets

Collecting blood and washing platelets were carried out in the same way as described for routine procedures. BCECF AM was dissolved in DMSO to make a 3 mM stock solution. 6 μM BCECF AM was added into 1 ml of platelet suspension (0.8-1 x 10^9 platelets/ml) and incubated at room temperature for 30 minutes (wrapped in tin foil or kept in the dark). 10 ng/ml of prostaglandin I_2 and 2 units of apyrase were present in this solution. The platelet suspension was spun at 550 xg for 10 minutes. Platelet pellet was resuspended in Tyrode's/Hepes platelet buffer and the concentration was adjusted to 2 x 10^8 platelets/ml.

F-2. Preparation of collagen matrix

Type I monomeric collagen separated from Vitrogen 100 collagen (1 mg/ml) was diluted with distilled water to a final concentration of 50 μg/ml. 100 μl of this collagen solution was used to coat the wells of a microtiter plate (NUNC, Kastrup, Denmark) for 90 minutes at room temperature. The collagen solution was removed by aspirating and rapping the plate sharply on a stack of paper towels. The wells then were blocked with 100 μl of 0.5% BSA solution in distilled water for 60 minutes at room temperature washed three times with PBS (pH 7.2).

F-3. Treatment of BCECF AM labeled platelets

Platelets were incubated with antibodies, and pharmacological inhibitors at room temperature for 30 minutres. 2 mM $MgCl_2$, 2 mM $CaCl_2$, 4 mM EDTA, or 4 mM EGTA were added to platelet suspension at the same time. Then 100 µl of these platelet suspensions were added to each well and incubated at room temperature for 45 minutes. The wells were washed three times with PBS again.

F-4. Quantitation of adherent platelets to collagen matrix

The platelets adhering to the collagen-coated wells were solubilized with 100 µl of 1% NP-40 platelet lysis buffer for 30 minutes to release intracellular BCECF. The fluorescence was read at an excitation wavelength of 490 nm, and an emission wavelength of 530 nm in a Perkin Elmer spectroflurometer 500 equipped with a microtiter plate reader. Control experiments were prepared by coating the wells with BSA. Although temperature may affect the BCECF fluorescence, this effect is probably too small to be of significance.

G. PLATELET ADHESION TO COLLAGEN BEADS UNDER STIRRING CONDITIONS

The method used for studying platelet adhesion to collagen beads was the same as described for aggregometry. The total number of platelets adhering to 100 beads at 30 seconds after the addition of collagen beads to the platelet suspension were randomly counted. Some mAbs and pharmacological inhibitors had significant effects on platelet adhesion to collagen beads. The percent control adhesion of platelets to the beads was calculated as follows: a). In the absence of mAbs or pharmacological inhibitors, the total number of platelets adhering to 100 beads (N_T) was used as a control of 100% adhesion. b). In the presence of mAbs or inhibitors, each of the total number of platelets adhering to 100 beads (N_E) was divided by N_T and multipled by 100. That is (N_E/N_T) x 100.

H. MEASUREMENT OF INTRACELULAR PH

H-1. Platelets labeling with BCECF AM

The procedure for BCECF AM loading platelets was the same as that described in F-1.

H-2. Measurement of pH_i

The experiments were performed using a Spex FluoroMax dm3000 spectrofluorometer (Rayonics Scientific Inc., Edison, NJ). The fluorescence was read at the wavelengths of excitation 490 nm and emission 530 nm. The number of BCECF-labeled platelets used in these experiments was 1 x 10^8 platelets/ml. The square cuvettes (ELKAY, Shrewsburt, MA) were used. Before beginning an experiment, the suspension of platelets was stirred for 1 to 2 minutes until the baseline stabilized. Then the agonists or inhibitors were added according to the experimental requirements. The change of pH_i was calculated based on the standard curve established.

I. CONTROLS AND STATISTICAL ANALYSIS

All studies of the monoclonal antibodies and pharmacological reagents were performed using time-paired untreated platelet controls. All experiments were performed on a minimum of three donors and at least in triplicate. Statistical differences between groups were evaluated by Student's t test.

CHAPTER III: RESULTS

A. PLATELET ACTIVATION BY TYPE I MONOMERIC COLLAGEN REQUIRES IMMOBILIZATION AND DIVALENT-CATIONS

A-1. Monomeric collagen is nonstimulatory until immobilized on latex beads

Monomeric collagen did not cause platelet aggregation (Fig.1, a), as previously described (13, 14, 15). However, we found that monomeric collagen immobilized on the surface of polystyrene latex beads did induce strong platelet aggregation (Fig.1, b). Collagen bead-induced aggregation could be blocked by soluble monomeric collagen in a dose-dependent manner (Fig.2), indicating that the immobilized signal did not depend upon a neo-conformation of the ligand on attachment to solid phase. Control experiment performed using BSA coated latex beads did not stimulate platelet aggregation (Fig.1, c), indicating that stirring with latex beads alone is not sufficient to generate an activating signal.

A-2. Platelet activation by type I monomeric collagen is dependent on divalent-cations

The aggregation of platelets induced by immobilized monomeric collagen was shown to be dependent on Mg^{2+} and inhibited by Ca^{2+} (Fig.3) as described for the adhesion of platelets to collagen under static conditions (18, 20, 91). Our results show that platelet adhesion to monomeric collagen under static conditions is dependent on Mg^{2+}, blocked by Ca^{2+}, and enhanced by removal of external Ca^{2+} with EGTA (Fig.11) indicating that Mg^{2+}, but not Ca^{2+} is absolutely required for platelet adhesion to collagen under static conditions. In contrast platelet adhesion to collagen beads under dynamic conditions has an absolute requirement for Ca^{2+} because it is dramatically blocked by chelating external Ca^{2+} with EGTA (Fig.15). These results suggest that platelet adhesion to collagen under dynamic and static conditions involves different mechanisms.

B. MECHANICAL FORCE CONTRIBUTES TO IMMOBILIZED COLLAGEN SIGNALS

When the continuous, nonthrombogenic layer of endothelium of the blood vessels is damaged, subendothelial elements, such as collagen, are exposed to the circulating blood and come into contact with platelets. Therefore, platelets are stimulated by contact with immobilized extracellular matrix (ECM) proteins of the vessel wall under conditions involving blood flow. To investigate whether the level of platelet-bead interaction determined the response to type I monomeric collagen we attached the collagen to polystyrene latex beads, and varied the stirring speed. As mentioned above, this type of collagen could not stimulate platelets in solution, but became an effective agonist on attachment to the beads. Platelets have to be stirred in order to aggregate. For soluble agonists such as ADP, and thrombin the level of platelet aggregation is closely related to stirring speed becoming maximal at about 900 rpm. The reasons why the extent of platelet aggregation is proportional to stirring speed is not clear, but may reflect a range of sensitivities to the frequency of contact stimulation. Platelet aggregation induced by bead-immobilized collagen was effective at much lower stirring speed, and increased with stirring speed plateauing at 500 rpm (Fig.4). In contrast collagen type I in fibrillar form was not effective at low stirring speed, and did not induce platelet aggregation until the stirring speed reached 700 rpm, and then levelled off at 900 rpm (Fig.4). Since bead-immobilized collagen would have greater mass and momentum than fibrillar collagen these findings are compatible with a mechanism involving mechanical force as a co-signal. Soluble agonists such as ADP, or thrombin induce platelets to bind to each other to form micro-aggregates. Interaction of micro-aggregates is probably an important factor in determining the rate of aggregation. Fibrillar collagen has very little mass, consequently it can only begin to aggregate platelets at relatively high stirring speeds. Monomeric type I collagen could not induce platelet aggregation even at stirring speeds of 1200 rpm. The response also increased with bead size from 0.8 to 4.22 μM diameter (Fig.5) compatible with the involvement of a mechanically sensitive signaling pathway. The possibility is that platelets would be stretched after spreading on the larger bead surface. In contrast platelets might be impossible to spread on the smaller bead surface. The force due to stretch may be different from shear force.

C. PLATELET MEMBRANE RECEPTORS INVOLVED IN THE RESPONSE TO COLLAGEN

C-1. Multiple receptors mediate immobilized collagen-induced platelet aggregation under dynamic conditions

Proposed platelet receptors for collagen, including GPIIb/IIIa, GPIa/IIa, and GPIV, have been investigated under static conditions. Few proteins have been identified under dynamic conditions. We show here that platelet aggregation induced by immobilized monomeric collagen is inhibited by mAb 4B4 directed against the β_1 integrin subunit (Fig.6) whereas the mAb had no effect on fibrillar type I collagen induced platelet aggregation (Fig.8). The responses were also inhibited by mAb AP1 directed against GPIb (Fig.6). Both receptors are connected to the cytoskeleton (5, 26, 162), a possible requirement for the response to the mechanical signals. Cytochalasin B, an inhibitor of actin polymerization, also blocked both immobilized collagen and fibrillar collagen-induced platelet aggregation confirming that rearrangement of the cytoskeleton is necessary for the collagen response (Fig.6). Platelet aggregation induced by collagen beads was dependent upon engagement of GPIIb/IIIa since it was inhibited by mAb P2 directed against GPIIb/IIIa, and by mAb SZ21 directed against GPIIIa. Anti-GPIa/IIa ($\alpha 2\beta 1$) mAb JBS-2 was found to completely block platelet aggregation indicating that GPIa/IIa is involved in recognition of collagen even though GPIa/IIa is only a minor membrane component (163). Anti-GPIV mAb 145-89 was unable to inhibit collagen-bead induced platelet aggregation (Fig.7), in contrast to Tandon's report on adhesion under static conditions suggesting that GPIV may be involved in platelet binding to collagen. MAb against GPIb interferes with Fc receptor signaling suggesting that the receptors are closely associated if not inter-dependent. In our experiments preincubation of the platelets with the anti-Fc receptor mAb IV.3 had no effect on immobilized collagen induced platelet aggregation (Fig.7), indicating that the Fc receptor (164) is not involved in the collagen signal.

CD63 is largely located in lysosomal granule membranes of non-activated platelets, increasing up to 100 times on the platelet surface after platelet activation (40, 165). The function of CD63 is not clear. We found that an anti-CD63 mAb 145-70 could block both collagen bead-induced and fibrillar collagen-induced platelet aggregation (Fig.7) which is the first evidence that CD63 may regulate platelet activation by collagen. Several reports have indicated that platelet adhesion to collagen is enhanced by vWf binding to GPIb (55, 166). In corroboration of these results we have found that platelet aggregation induced by immobilized collagen and fibrillar collagen was blocked by the anti-vWf mAb ESvWf7 (Fig.7) suggesting that vWf binding might be required for collagen-mediated aggregation. vWf has been previously reported to be important for platelet adhesion to collagen since vWf enhances binding through interaction with GPIb (54).

C-2. Integrins mainly involve platelet adhesion to collagen beads under dynamic conditions (Fig.9)

Platelet adhesion to damaged blood vessel wall is a rapid event (167), but the process under static conditions takes minutes or even hours (17, 19, 20). The mechanism remains unclear. Little is known about the early events in platelet adhesion to the immobilized collagen of blood vessel wall. To investigate platelet adhesion under dynamic conditions we preincubated platelet suspension with mAbs and measured platelets adhering to the collagen beads at 30 seconds after adding the collagen beads. MAb JBS-2, mAb P2, and mAb 4B4, directed against integrin subunits $\alpha 2\beta 1$, $\alpha IIb\beta 3$, and $\beta 1$ respectively, very effectively inhibited (over 95%) platelet adhesion to the beads indicating that these integrins were involved in the early stages of platelet adhesion to collagen.

The anti-GPIb mAb AP1 inhibited platelet adhesion to the beads by 37% suggesting that GPIb may be partially involved in the initial step of platelet adhesion to collagen. Anti-GPIV mAb 145-89, anti-CD63 mAb 145-70, and anti-vWf mAb ESvWf7 had little effect on the early stage of attachment excluding the possibility that these components were involved in the early process. Overall, integrins played a far more important role in the early event of platelet adhesion to collagen beads under dynamic condition than other non-integrin proteins did.

C-3. Several receptors involve platelet adhesion to collagen matrix under static conditions (Fig.9)

As mentioned above, platelet adhesion to collagen has been studied widely under static conditions. We also compared the receptors involved in platelet adhesion to collagen under dynamic conditions with those involved in platelet adhesion to collagen matrix under static conditions. Adhesion was strongly blocked by mAb JBS-2, an anti-GPIa/IIa mAb, and by mAb 145-89, an anti-GPIV mAb indicating GPIa/IIa and GPIV were both involved in collagen binding. MAb P2, which recognizes the GPIIb/IIIa complex, enhanced platelet attachment to collagen under static conditions by more than 2 fold which is very different from previous reports (19), and may be due to differences in time-course and the assays employed. mAb 4B4, which is specific for the $\beta 1$ integrin subunit, also enhanced the binding by over 2 fold implying that the $\beta 1$ integrin subunit may downregulate platelet attachment to collagen under static conditions. GPIb has been reported to regulate platelet adhesion to collagen (55, 168). We therefore investigated whether mAb AP1, an anti-GPIb mAb, could affect platelet adhesion to collagen. MAb AP1 only inhibited the binding by 20% while mAb AP1 could block collagen bead-induced platelet aggregation by 95% (Fig.6) indicating that GPIb is not critical for binding, but plays an important role in the granule release. Anti-vWf mAb ESvWf7 and anti-CD63 mAb 145-70 had little effect on the binding of platelets to collagen under static or dynamic conditions indicating that they do not affect platelet adhesion to collagen, but rather platelet aggregation.

D. DIFFERENT SIGNALING PATHWAYS ARE INVOLVED IN PLATELET ACTIVATION BY COLLAGEN UNDER DIFFERENT CONDITIONS

D-1. The involvement of PLA2 and PLC pathways in collagen bead-induced platelet aggregation

Following platelet activation by collagen the activities of PLA2 and PLC are stimulated (98, 99, 100, 101, 102). Both activation of phospholipases A2 and C is dependent on endogenously generated thromboxane A2 (TXA2)(100, 107, 169). To understand the signaling pathways involving in collagen bead-induced platelet aggregation we used a panel of pharmacological inhibitors and found evidence that both PLA2 and PLC are necessary in this process.

The PLA2 inhibitor BPB blocked platelet aggregation induced by both immobilized collagen and fibrillar collagen at levels known to inhibit PLA2 activity (Fig.12 & 16) indicating the possible involvement of PLA2 in both. Since Na^+/H^+ exchange was suggested to be involved in regulating PLA2 activity (116), we investigated the effect of the Na^+/H^+ exchange blocker HMA or EIPA on the collagen-induced signal. We found that they could block platelet aggregation induced by immobilized collagen (Fig.14), but could not block the response induced by fibrillar collagen (Fig.16). This implies that the involvement of Na^+/H^+ exchange is critical in signal transduction by immobilized collagen. However, activation of Na^+/H^+ exchange as evidenced by cytoplasmic alkalinization was not observed with either immobilized collagen or fibrillar collagen (data not shown). This may reflect localized pH change too small to measure in whole platelets. The response of platelets to immobilized monomeric collagen is similar in this respect to that described for the soluble agonists epinephrine and ADP which also require Na^+/H^+ exchange to activate PLA2 upstream of PLC (108, 116), and to the mechanism of platelet activation by anti-CD9 mAb (158).

Indomethacin, a cycloxygenase inhibitor, could completely block platelet aggregation induced by immobilized collagen and fibrillar collagen (Fig.13 & 17) indicating that thromboxane synthesis is absolutely required. NDGA, an inhibitor of lipoxygenase, blocked collagen bead- and fibrillar collagen-induced platelet aggregation by less than 30% (Fig.13) indicating the lipoxygenase pathway is less important in collagen response. Thromboxane receptor inhibitor BM13.177 reduced platelet aggregation to immobilized collagen by over 85% (Fig.13), but inhibited the response to fibrillar collagen by less than 40% (Fig.17). This suggests that thromboxane interaction with its receptor is more important for the immobilized collagen signal. Genistein, a protein tyrosine kinase inhibitor (170), blocked collagen-bead-induced platelet aggregation (Fig.13) indicating that tyrosine kinase activity may be important for the PLA2 activation required for immobilized collagen-induced platelet aggregation.

Neomycin, a PLC inhibitor, blocked both collagen bead- and fibrillar collagen-induced platelet aggregation (Fig.12), suggesting the involvement of PLC pathway in collagen signal. Collagen-induced platelet activation may involve PKC since it is sensitive to the PKC inhibitor sphingosine (105). Our experiments (Fig.12) confirmed that sphingosine could block either immobilized or fibrillar collagen-induced platelet aggregation. However, sphingosine is also a calmodulin-dependent kinase inhibitor (171) and may be blocking collagen signaling through the calmodulin pathway. W7, a calmodulin-dependent kinase inhibitor, could block collagen response (Fig.12), supporting a role for calmodulin in mediating platelet aggregation. Although staurosporine, a PKC inhibitor, did not inhibit fibrillar collagen or the immobilized collagen-induced response we cannot exclude the possibility of that PKC is involved in the collagen signal since the concentration of staurosporine used may be too low to block PKC.

D-2. The involvement of PLA2 and PLC pathways in platelet adhesion to collagen beads under dynamic conditions (Fig.10)

The method used for this study was same as described in C-2. Adhesion to the beads was examined under a microscope. We preincubated pharmacological inhibitors with platelets for 3 minutes, and then the collagen beads were added. The number of platelets bound to the surface of the beads were counted at 30 seconds after adding the beads to the platelet suspension. We found that all inhibitors except BM13.177, NDGA, and staurosporine could inhibit platelet binding to the surface of the collagen beads. Among these inhibitors, genistein, HMA, BPB, and neomycin were the most powerful and could block platelet adhesion to the beads by over 90%. Indomethacin, W7, and sphingosine also inhibited binding by 70-80%. These data suggest that both PLA2 and PLC activation are required for initial adhesion of platelets to the beads.

D-3. The involvement of PLA2 and PLC pathways in platelet adhesion to collagen matrix under static conditions (Fig.11)

In chapter II, we described the adhesion assay which was used to study platelet adhesion to collagen-coated wells under static conditions. Our results suggest that the signaling pathways involved in platelet adhesion to collagen matrix under static conditions are the same as those involved in platelet adhesion to collagen beads under dynamic conditions. However, HMA and BPB caused less inhibition of platelet adhesion to collagen matrix under static conditions, suggesting that the activation of Na^+/H^+ exchange and PLA2 is partially required for the early stage of platelet adhesion to collagen.

In summary, our results provide evidence of fundamental similarities in the response to fibrillar collagen, bead-immobilized collagen, and plastic-immobilized collagen, but also identify some dramatic differences. Platelet adhesion to monomeric collagen type I under dynamic conditions has an absolute requirement for interaction with the α chain of the $\beta 1$ integrin $\alpha 2\beta 1$, and for the activation of phospholipase A2 (PLA2). Platelet adhesion to monomeric collagen under static conditions partially requires activation of PLA2. These results suggest that mobilization of arachidonic acid through PLA2 activation may be necessary for maintaining high avidity interactions with collagen under both conditions. Since an anti-$\beta 1$ integrin mAb could block adhesion to monomeric collagen under dynamic conditions but not under static conditions, the β1 integrin subunit may play a role in the differential coupling of α2β1 to Na^+/H^+ exchange. Platelet adhesion to monomeric collagen under static conditions is dependent on Mg^{2+}, blocked by Ca^{2+}, and enhanced by removal of external Ca^{2+} with EGTA. However, platelet adhesion to collagen beads under dynamic conditions has an absolute requirement for Ca^{2+} because it is blocked by chelating external Ca^{2+} with EGTA. Since an anti-β3 integrin mAb could block platelet adhesion to collagen beads under dynamic conditions, but not under static conditions, the β3 integrin may play a role in the coupling of αIIbβ3 to importation of Ca^{2+}. Therefore, these results suggest that force-dependent PLA2 activation by importation of Ca^{2+} through αIIbβ3 is necessary for platelet adhesion under dynamic conditions. Since cytoplasmic alkalinization is a mechanism favoring the activation of PLA2 the apparent dependency of bead-induced aggregation on Na^+/H^+ exchange and the lack of this dependency upon stimulation by fibrillar collagen suggests that the latter directs an alternative way to activate PLA2. The lack of effect of the anti-β1 integrin mAb on platelet aggregation by fibrillar collagen is also in accordance with the lack of Na^+/H^+ exchange-dependence. Similarly the involvement of GPIV in the adhesion of platelets to monomeric collagen under dynamic and static conditions suggests the existence of both force-dependent and force-independent signaling mechanisms.

CHAPTER IV: DISCUSSION

Monomeric collagen. Although native monomeric collagen does not induce platelet aggregation in solution (13, 14, 15, 16, 50, 172), monomeric collagen can efficiently support platelet adhesion (17). By attaching collagen to latex beads, we showed that immobilized monomeric collagen could become a potent agonist of platelet aggregation if the beads were stirred. The need for stirring suggest a requirement for mechanical force. Although we cannot absolutely exclude the possibility that some monomeric collagen might polymerize on contact with the beads a number of lines of evidence argue against this: First, care was taken to load and wash the beads under acid conditions (pH 3.5). Second, collagen solutions were separated by passing through a 300 kDa filter to exclude any fibrillar collagen. Third, same type of collagen in soluble form could block immobilized collagen-induced platelet aggregation, but not aggregation induced by fibrillar collagen indicating that signal from immobilized monomeric collagen could not be ascribed to neo-conformation of the ligand on attachment to a solid phase. If some monomeric collagen detached from the collagen beads and formed fibrillar collagen in the suspension, this solution without beads should have induced platelet aggregation like fibrillar collagen. These findings suggest that activation by the beads is really due to monomeric collagen bound on the surface of the beads. The functional study also provides evidence to support this conclusion. For example, the Na^+/H^+ exchange blocker HMA could block platelet aggregation induced by immobilized collagen, but could not block fibrillar collagen-induced platelet aggregation. In conclusion, immobilized monomeric collagen appears to generate stimulatory signals on platelets capable of inducing platelet aggregation. This signaling appears to involve immobilization and the application of mechanical force. Therefore, immobilized monomeric collagen may provide a simple and homogeneous signal capable of inducing platelet aggregation.

Platelet adhesion. As previously discussed, mechanical forces play an important role in regulating platelet function. In order to characterize the mechanism of platelet activation by immobilized collagen and to assess the role of mechanical force in collagen-platelet contact signaling, we compared platelet adhesion to bead-immobilized collagen under dynamic conditions with that to plastic plate-immobilized collagen under static conditions. We found that platelet adhesion to collagen under both dynamic and static conditions was dramatically different although there are some similarities. Platelet adhesion to collagen beads under dynamic conditions or to collagen matrices under static conditions was strongly blocked by the anti-$\alpha 2\beta 1$ integrin mAb JBS-2 indicating that $\alpha 2\beta 1$ integrin is absolutely required in mediating platelet adhesion to collagen under both conditions. MAb P2 and mAb 4B4, directed against $\alpha IIb\beta 3$ and $\beta 1$ integrins respectively, also profoundly inhibited platelet adhesion to collagen beads under dynamic conditions. In contrast, they did not block, but actually enhanced platelet adhesion to collagen matrices under static conditions suggesting that different signals are involved. HMA, an inhibitor of the Na^+/H^+ exchanger, and BPB, an inhibitor of PLA2, inhibited platelet adhesion to collagen under both dynamic and static conditions indicating that Na^+/H^+ exchange-dependent activation of PLA2 might be required to affect adhesion under both conditions. Removal of external Ca^{2+} with EGTA was shown to significantly inhibit platelet adhesion to collagen beads suggesting importation of Ca^{2+} which in turn activated PLA2 might be critical in this process. In Fig.18, we propose a model of the mechanism of platelet activation by collagen which is consistent with our results.

The role of Na^+/H^+ exchange-coupled PLA2 activation through β1 integrin in platelet adhesion. Taken together, our results suggest that α2 and β1 integrin subunits possess separate functions in regulating platelet adhesion to collagen since mAb against the α2β1 integrin could block platelet adhesion to collagen under both conditions, but mAb against the β1 subunit could only block platelet adhesion to collagen beads under dynamic conditions. Integrin β1, the transmembrane ECM receptor, may act as a force-transducer since it can transmit mechanical signals to the cytoskeleton (26). Tyrosine phosphorylation of a 125 kDa focal adhesion kinase has been proposed as a transducer of integrin-generated signaling pathways (6, 7, 173). The importance of tyrosine phosphorylation in platelet activation is supported by our findings that the tyrosine kinase inhibitor genistein blocked platelet adhesion. Cytoplasmic alkalinization (6) and the activation of PLA2 may occur downstream of integrin-mediated tyrosine phosphorylation. Cytosolic alkalinization favorably affects the activity of PLA2 and other enzymes (174). Other workers in our laboratory have shown that both the Na^+/H^+ exchange blocker HMA and the PLA2 inhibitor BPB cause profound inhibition of the formation of thromboxane A2. The inhibitory effect of BPB on platelet adhesion to collagen may be overcome by the addition of arachidonic acid (data not shown), suggesting Na^+/H^+ exchange-mediated release of arachidonic acid through receptor-coupled PLA2 (116) may play a major role in regulating platelet adhesion to collagen. It was recently reported that an anti-$\beta 1$ integrin antibody could also enhance collagen-mediated elevation of arachidonic acid in a kidney cell (175). This is the first report of a connection between arachidonic acid and adhesion to native collagen. Released arachidonic acid from membrane phospholipids cleaved by PLA2 has been reported to induce cell spreading (119). These results are compatible with our observation that anti-$\beta 1$ integrin mAb 4B4 enhanced platelet adhesion to collagen under static conditions. However, with the application of mechanical agitation, both inhibition of Na^+/H^+ exchange-mediated release of arachidonic acid through receptor-coupled PLA2 and occupation of the $\beta 1$ integrin subunit could block platelet adhesion to collagen beads, suggesting that either of these components is absolutely required in this process. In addition, because the integrins of the β_1 (or VLA) subfamily may share a common β_1 subunit, a mAb (4B4) directed against a common β_1 epitope could affect all of these integrins including $\alpha_5\beta_1$ and $\alpha_v\beta_1$, the fibronectin receptors, $\alpha_6\beta_1$, the laminin receptor, $\alpha 1\beta 1$ and $\alpha 2\beta 1$, the receptors for collagen, and $\alpha_3\beta_1$, the receptor for fibronectin, laminin, and collagen (5). Our data make an important distinction between the mechanisms of platelet activation under dynamic and static conditions. We propose that the ability of platelets to bind to collagen under dynamic conditions is dependent upon Na^+/H^+ exchange-mediated PLA2 activation through the β1 integrin subunit. In contrast under static conditions PLA2 activation is directly mediated by the Na^+/H^+ exchange without the involvement of the β1 integrin subunit. PLA2 activation may in turn convert the β1 integrin to a high affinity state for platelet adhesion to collagen. If the role of the exchanger is to lower the threshold for activation of cytosolic PLA2 then an attractive possibility is that platelet adhesion under dynamic conditions may involve force-dependent activation of the exchanger by tyrosine phosphorylation of the 125 kDa protein ($pp125^{fak}$) through the activation of the β1 integrin subunit. Under static conditions, however, activation of the exchanger may involve an alternate mechanism independent of the β1 integrin subunit. In conclusion our findings support the possibility that β1 integrin may directly participate in the regulation of PLA2 activity through coupling to the Na^+/H^+ exchanger under dynamic conditions, perhaps by acting as a force-transducer (26).

The role of Ca^{2+} importation-coupled PLA2 activation through β3 integrin in platelet adhesion. Since intracellular Ca^{2+} is an important co-requirement for the activation of PLA2, we speculated that the force-dependent pathway might couple the activity of the Na^+/H^+ exchanger to Ca^{2+} importation. As previously discussed GPIIb/IIIa has been shown to be associated with a Ca^{2+} channel or to act as a Ca^{2+} channel. This suggested to us that the ability of mAb P2, directed against $\alpha IIb\beta 3$ integrin, to block platelet adhesion only under dynamic conditions, might reflect an effect on Ca^{2+} importation. To test whether external Ca^{2+} is differentially required for adhesion under dynamic and static conditions we investigated the effect of chelating external Ca^{2+} with EGTA. Our finding that EGTA prevented platelet adhesion to collagen beads under dynamic conditions, but enhanced binding under static conditions supports this possibility. Under static conditions PLA2 may be activated by a mechanism which does not require the importation of Ca^{2+} since adhesion was enhanced by the removal of external Ca^{2+} with EGTA. Crosslinking GPIIb/IIIa enhanced binding, suggesting that this receptor might also be involved in regulating platelet binding to collagen in a manner independent of Ca^{2+}. Our findings that both Ca^{2+} importation and the β3 integrin are involved in platelet adhesion to the beads under dynamic conditions is novel. In this situation, the elevation of intracellular Ca^{2+} through the β3 integrin appears sufficient to trigger the activation of PLA2. Arachidonic acid released through PLA2 activation may contribute to platelet adhesion to collagen (176). Removal of external Ca^{2+} and blockade of GPIIb/IIIa function appear to completely eliminate Ca^{2+} importation across the plasma membrane, thus blocking platelet adhesion to the collagen-beads. GPIIb/IIIa has been previously reported to be involved only in the late events of platelet activation (19, 20), however, those experiments in contrast to ours were carried out under non-physiological conditions with platelet aggregation being induced by fibrillar collagen, rather than by immobilized collagen, and in which platelet adhesion taking place under static conditions over 45 to 90 minutes rather than within seconds as in our dynamic model. However, our results under static conditions are also different from those of other investigations (19). We found that platelet adhesion to type I monomeric collagen was enhanced by crosslinking of GPIIb/IIIa by mAb P2. In contrast, they showed that mAb P2 blocked platelet adhesion to type III fibrillar collagen. The different collagens might generate different signals. Activation of transmembrane ECM receptors, such as $\alpha IIb\beta 3$ integrin, is accompanied by conformational changes affecting their adhesive features (161). Removal of Ca^{2+} by EGTA from the putative divalent cation-binding sites on GPIIb/IIIa may result in a conformational change and loss of GPIIb/IIIa receptor function (82). The altered conformation of GPIIb/IIIa may be reorganized by the crosslinking of GPIIb/IIIa by mAb P2 under static conditions over a longer time-frame. Thus, we postulate that monomeric collagen, but not fibrillar collagen may complementarily bind to the GPIIb/IIIa complex to generate enhanced signal. In addition, Mg^{2+} may compete with Ca^{2+} on the same binding site to exert the same effect as EGTA did. Furthermore, in the presence of Ca^{2+}, it may contribute to the stabilization of GPIIb/IIIa conformation and the maintenance of GPIIb/IIIa function. Under these conditions, blockade of GPIIb/IIIa would still have an inhibitory effect on platelet adhesion to collagen. However, under dynamic conditions the conformation of GPIIb/IIIa may not be changed by varying the divalent-cation concentration and the function of GPIIb/IIIa would remain unchanged.

The role of GPIV in platelet adhesion. Non-integrin proteins may also be coupled to mechanical force to modulate platelet adhesion to collagen. For example, our anti-GPIV mAb 145-89 very significantly inhibited platelet adhesion to collagen under static conditions. This is consistent with Tandon's experiments with a polyclonal antibody against GPIV supporting a function for GPIV as a collagen receptor (74). However, mAb 145-89 had no effect on adhesion to collagen under dynamic conditions in our experiments, suggesting that stirring may bypass the requirement for GPIV. Our mAb 145-89 may recognize a specific epitope on GPIV in contrast to Tandon's antibody which is polyclonal and may recognize a number of epitopes(74). Since GPIV has also been proposed as a receptor for thrombospondin (79, 80) it is unclear whether the effects observed by Tandon are specific for collagen-platelet interaction. MAb145-89 in contrast may specifically block platelet-collagen interaction. However, the effect of mAb 145-89 on thrombospondin-mediated binding is unknown. A role for GPIV in regulating platelet adhesion to collagen is also suggested by the fact that Nak^a-negative platelets (75) show reduced binding to collagen both under Mg^{2+}-free conditions and at very early time points in the presence of Mg^{2+}. These results contrast with our observation that anti-GPIV mAb 145-89 could almost completely block platelet adhesion to collagen under static conditions. One possible reason for this discrepancy is that mAb 145-89 may recognize an epitope on GPIV involved in binding to both collagen and thrombospondin. Another possibility is that the Nak^a-negative platelets may in fact express a small number of GPIVs which, though too small to detect, may nevertheless be capable of mediating platelet adhesion to collagen under static condition over a period of time. It is also possible that mAb145-89 recognizes an epitope on GPIV specific for collagen, thereby accounting for its ability to completely block the ability of GPIV to mediate platelet adhesion to collagen. GPIV (CD36) has been shown to be a signal transduction molecule. The $pp60^{fyn}$, $pp62^{yes}$, and $pp54/58^{lyn}$ protein tyrosine kinases of the src gene family are tightly associated with CD36 in nonactivated platelets (132). Complexes of CD36 and these protein tyrosine kinases are not found after anti-GPIV antibodies have been used in activated platelets (133). Therefore, the lack of these protein tyrosine kinases in activated platelets may result in the blockade of signal from the activation of GPIV. We speculate that mAb 145-89 had no effect on platelet-collagen interaction under dynamic conditions because the effect of src-related kinases may be bypassed under dynamic conditions where platelets are activated quickly; in contrast src-related kinases may not be bypassed under static conditions where platelets are activated slowly behind the inhibitory effect of anti-GPIV mAb 145-89.

Platelet aggregation. Although considerable investigation has been performed on characterizing specific receptors for collagen, little is known about how the receptors transduce signals across the membrane to initiate platelet aggregation. However, platelet activation will not occur unless platelets are stirred, or shear stress is applied (10, 11, 21). It is difficult to differentiate between mechanical force and contact events. Our model attemps to simulate *in vivo* the hemodynamic conditions by attaching monomeric collagen to polystyrene latex beads and bringing the beads into contact with platelets under stirring conditions to investigate the role of locally applied mechanical stress in collagen-induced aggregation. We found that monomeric collagen in solution could not induce platelet aggregation even when the stirring speed exceeded 1200 rpm, whereas platelet aggregation induced by immobilized monomeric collagen increased with stirring speed plateauing at 500 rpm. Although fibrillar collagen is a powerful agonist, it could not induce platelet aggregation until the stirring speed reached 700 rpm. Platelet aggregation increased with bead size from 0.8 to 4.22 μM in diameter also suggesting the involvement of a mechanically-dependent signaling pathway since platelet spreading on the larger bead-surface might result in more platelet spreading. Therefore, our results suggest that mechanical force may play a significant role in collagen-mediated platelet aggregation, and that it might regulate integrins, Na^+/H^+ exchange , and PLA2 to generate activating signals. Platelet aggregation-induced by immobilized monomeric collagen required thromboxane synthesis, an active Na^+/H^+ exchanger, and the involvement of the β_1 integrin subunit. In contrast platelet aggregation by fibrillar collagen did not require Na^+/H^+ exchanger, was not inhibited by mAb 4B4, and was less dependent on thromboxane synthesis. Thromboxane is synthesized from arachidonic acid released from membrane phospholipids by PLA2 and interacts with a GTP-associated receptor. BPB, a PLA2 inhibitor, blocked platelet aggregation induced by collagen beads and fibrillar collagen indicating the involvement of a common signaling pathway. Both modes of platelet aggregation were blocked by indomethacin, a cycloxygenase inhibitor, suggesting that thromboxane synthesis was absolutely required. An inhibitor of lipoxygenase, NDGA, blocked platelet aggregation by less than 30% implying that this pathway is less important in the collagen response. BM13.177, a thromboxane receptor inhibitor (160), could block platelet aggregation induced by immobilized collagen and fibrillar collagen, but it blocked platelet aggregation induced by immobilized collagen by over 85%, and the response to fibrillar collagen by less than 40%, indicating that thromboxane interaction with its receptor was more important for immobilized collagen signal. Thromboxane synthesis involves a synergy between signals from collagen receptors and thromboxane receptor (101) which does not require Na^+/H^+ exchange in fibrillar collagen-mediated platelet activation (131). Previous work has shown that the Na^+/H^+ exchanger regulates receptor-mediated PLA2 activation in ADP or epinephrine stimulated human platelets (108). We therefore used the Na^+/H^+ exchange blockers HMA and EIPA to investigate the effect of the Na^+/H^+ exchanger on platelet aggregation induced by immobilized collagen and fibrillar collagen. The response of platelets to bead-immobilized monomeric collagen was dependent upon Na^+/H^+ exchange which is similar in this respect to that described for the soluble agonists ADP and epinephrine which also need Na^+/H^+ exchange to activate PLA2 upstream of PLC (108, 116) and to the mechanism of platelet activation by anti-CD9 mAb (158). We also investigated the effect of Na^+/H^+ exchange on collagen-induced eicosanoid mobilization. Inhibition of Na^+/H^+ exchange resulted in strong inhibition of collagen bead-induced TXA2 formation (data not shown), further suggesting that Na^+/H^+ exchange is a prerequisite for this process. Our results therefore suggested that PLA2 was activated by monomeric collagen by a mechanism which required immobilization of the stimulus, as well as Na^+/H^+ exchange, and that this differed from the activation of PLA2 by fibrillar collagen. Since the alkalinization of intracellular pH in spread fibroblasts is thought to be regulated by clustering β_1-integrin that enhances the transmembrane Na^+/H^+ exchange (29, 30), our finding that activation by monomeric collagen, but not by fibrillar collagen could be inhibited by anti-β_1 integrin mAb would be compatible with a mechanism coupling β_1 integrin modulation of Na^+/H^+ exchange to the activation of PLA2. In conclusion, collagen bead-induced platelet aggregation was mediated by Na^+/H^+ exchange-coupled PLA2 activation probably through clustering of $\beta 1$ integrin.

Collagen receptors. The unique finding that immobilized monomeric collagen could induce platelet aggregation may widen the scope of our investigation on the nature of the collagen-induced signal transduction. Although receptors for collagen have been extensively studied as described above, little is known about the function of these receptors in mediating platelet activation, especially platelet aggregation by monomeric collagen. In order to demonstrate the involvement of these receptors in regulating platelet activation, several mAbs were used. Anti-$\alpha_2\beta_1$ mAb was profoundly inhibitory for platelet aggregation and adhesion as suggested by other researchers (17, 19, 20, 91, 92), suggesting that $\alpha 2\beta 1$ is absolutely required for platelet adhesion and aggregation. Although there is no evidence that GPIb may serve directly as a collagen receptor, many reports have demonstrated that platelet adhesion to collagen is enhanced by vWf binding to GPIb (55, 56). Our results suggest that GPIb is involved in platelet aggregation, a late event in platelet activation, but only partially participates in the early stages of platelet adhesion to collagen because anti-GPIb mAb AP1 almost completely blocks platelet aggregation induced by both monomeric and fibrillar collagen, and has only minor effects on platelet adhesion under either dynamic or static conditions. Our results are consistent with those of Ruan (134) who showed that anti-GPIb mAb SZ2 blocked collagen-induced platelet aggregation, but not adhesion, suggesting that an epitope recognized by both mAb SZ2 and mAb AP1 might not be critical for rapid platelet adhesion. Ruan also showed that mAb AN51, which completely blocked the binding of vWf to platelets, had no effect on collagen-induced platelet aggregation, suggesting first that the inhibition of the binding of vWf to platelets by mAb SZ2 was unlikely to represent the mechanism by which SZ2 inhibited collagen-induced platelet aggregation, and second that the function of one collagen-binding site might be influenced by proximity to GPIb in the platelet membrane. Anti-vWf mAb ESvWf7 had little effect on platelet adhesion to collagen under either dynamic or static condition, indicating that vWf was not involved in the early stage of platelet activation. MAb ESvWf7 could almost completely block platelet aggregation, suggesting that the platelet interaction with membrane-surface-bound vWf might play a major role in platelet aggregation. However, no direct information is available for the mechanism of vWf-mediated platelet aggregation although the interaction of GPIb with vWf under shear force is involved in platelet adhesion (10, 11, 135). Anti-CD63 mAb 145-70 could inhibit platelet aggregation by 95%, and had little effect on platelet adhesion indicating that after the fusion of lysosomal membrane with plasma membrane upon platelet activation, anti-CD63 mAb could exert its inhibitory effect on platelet aggregation. Because of limited knowledge of CD63, it is difficult to interpret how CD63 is involved in platelet activation. Further investigation has to be done in this novel area. As discussed above, $\alpha_{IIb}\beta_3$, β_1, and GPIV might be related to mechanical force to regulate platelet function. In addition, GPIb, $\alpha_{IIb}\beta_3$, and β_1 integrin subunits are connected to the cytoskeleton (5, 162, 144), a possible requirement for the response to the mechanical signals. The inhibitor of actin polymerization cytochalasin B blocked both immobilized collagen and fibrillar collagen-induced platelet aggregation suggesting that rearrangement of cytoskeleton is necessary for the collagen signal.

CHAPTER V: CONCLUSION

The objective of this thesis was to characterize the mechanism of platelet activation by immobilized monomeric collagen and to assess a possible role for mechanical force in collagen-induced contact signaling. The work was based on the premise that immobilized soluble monomeric collagen on latex beads when brought into contact with platelets by stirring mimics some of the physical requirements for platelet activation by ECM contact *in vivo*.

The presentation of monomeric collagen on solid phase for the first time provides a way to investigate the signals stimulated by immobilized monomeric collagen under conditions of platelet suspension. As hypothesized, the requirements for platelet aggregation by immobilized collagen and fibrillar collagen are indeed different. Evidence is presented that platelet aggregation-induced by immobilized monomeric collagen requires thromboxane synthesis, an active Na^+/H^+ exchanger, and the involvement of the β_1 integrin subunit. In contrast platelets activated by fibrillar collagen do not require Na^+/H^+ exchange, are not blocked by mAb against the β_1 integrin subunit, and are less dependent on thromboxane synthesis. Our results are compatible with the possibility that platelets stimulated by monomeric collagen activate PLA2 by a pathway involving Na^+/H^+ exchange, and the β_1 integrin subunit. Since platelets activated by fibrillar collagen could be inhibited by the PLA2 inhibitor BPB they may activate PLA2 by a different mechanism. Therefore, our results provide evidence of some dramatic differences between monomeric and fibrillar collagens although there may be some overlaps in their mechanisms.

Mechanical force was shown to be involved in platelet activation by collagen. The association of mechanical force with integrins, Na^+/H^+ exchange, and PLA2 may critically drive the collagen signal. mAbs directed against a $\beta 1$ integrin could block platelet adhesion to collagen beads under dynamic conditions and enhance platelet adhesion to collagen matrices under static conditions. In contrast anti-$\alpha 2\beta 1$ mAbs block platelet adhesion under both conditions. Na^+/H^+ exchange, and PLA2 were shown to be absolutely required in regulating platelet adhesion to collagen under both dynamic and static conditions. In agreement with Schwartz's report that clustering of the $\beta 1$ integrin results in the activation of Na^+/H^+ exchange (30), we conclude that platelet adhesion mediated by force-dependent PLA2 activation requires stimulation of Na^+/H^+ exchange through the $\beta 1$ integrin. mAb directed against the $\alpha IIb\beta 3$ integrin had the same effect on platelet adhesion to collagen as anti-$\beta 1$ integrin mAb did. Chelation of external Ca^{2+} by EGTA profoundly inhibited platelet adhesion to collagen beads under dynamic conditions, but enhanced platelet binding to collagen matrices under static conditions. GPIIb/IIIa ($\alpha IIb\beta 3$) has been implicated in the process of Ca^{2+} influx which in turn activates PLA2 activity. Therefore the fact that platelet adhesion to collagen beads under dynamic conditions was prevented by removal of external Ca^{2+} with EGTA suggests that platelet adhesion mediated by force-dependent PLA2 activation also requires Ca^{2+} importation through the $\beta 3$ integrin. We believe that the involvement of the αIIbβ3 integrin in regulating an early stage of platelet adhesion to collagen under dynamic conditions is a unique finding which will contribute to the better understanding of integrins in mediating cell adhesion to ECM.

The possible platelet collagen receptors have also been studied. Several mAbs against platelet membrane glycoproteins were used to investigate the involvement of these receptors in the collagen activation signal. As discussed in chapter IV, α2β1, β1, β3, αIIbβ3 integrins, GPIV, GPIb, and CD63 are implicated in platelet activation by collagen.

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Figure 1

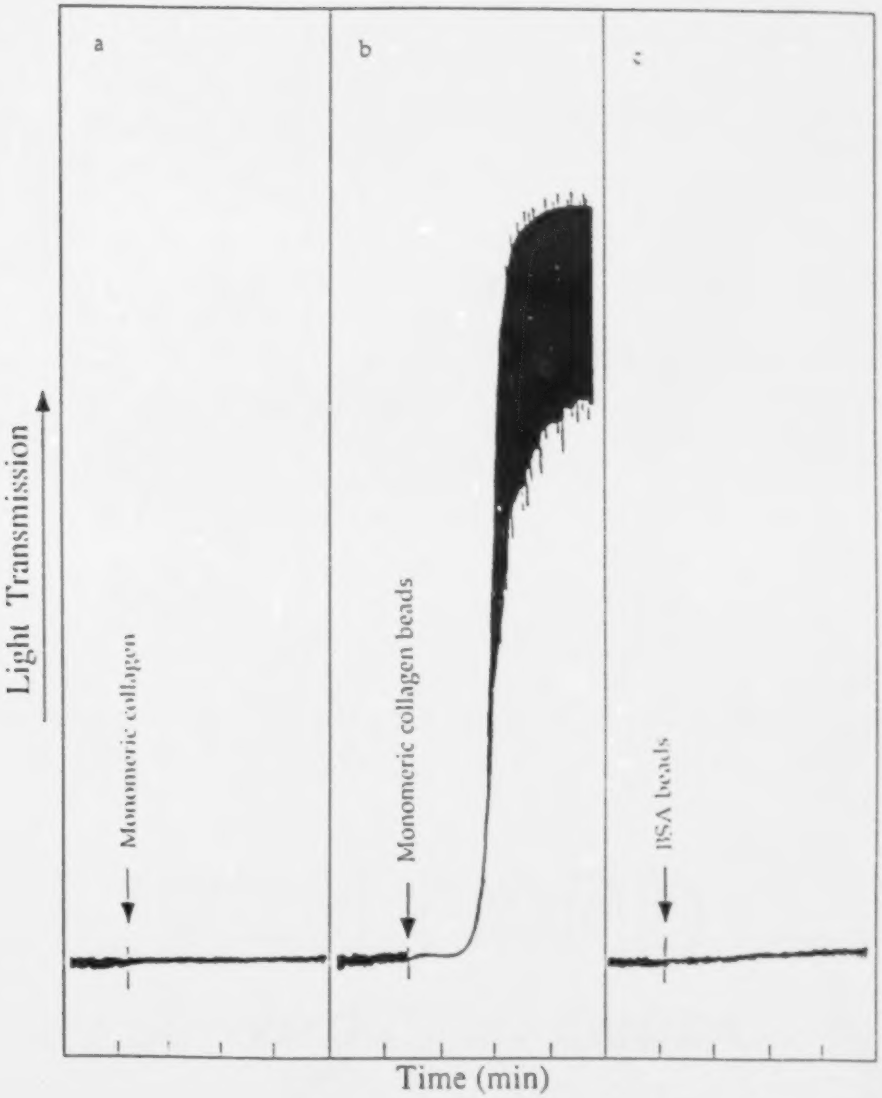


Fig.1: *Immobilized monomeric collagen: a potent agonist for platelet aggregation.* Monomeric type I collagen (up to 10 μg/ml) could not induce platelet aggregation (a), unless immobilized on the surface of a polystyrene latex bead (b). Attachment of BSA on the latex beads did not stimulate platelets (c).

Figure 2

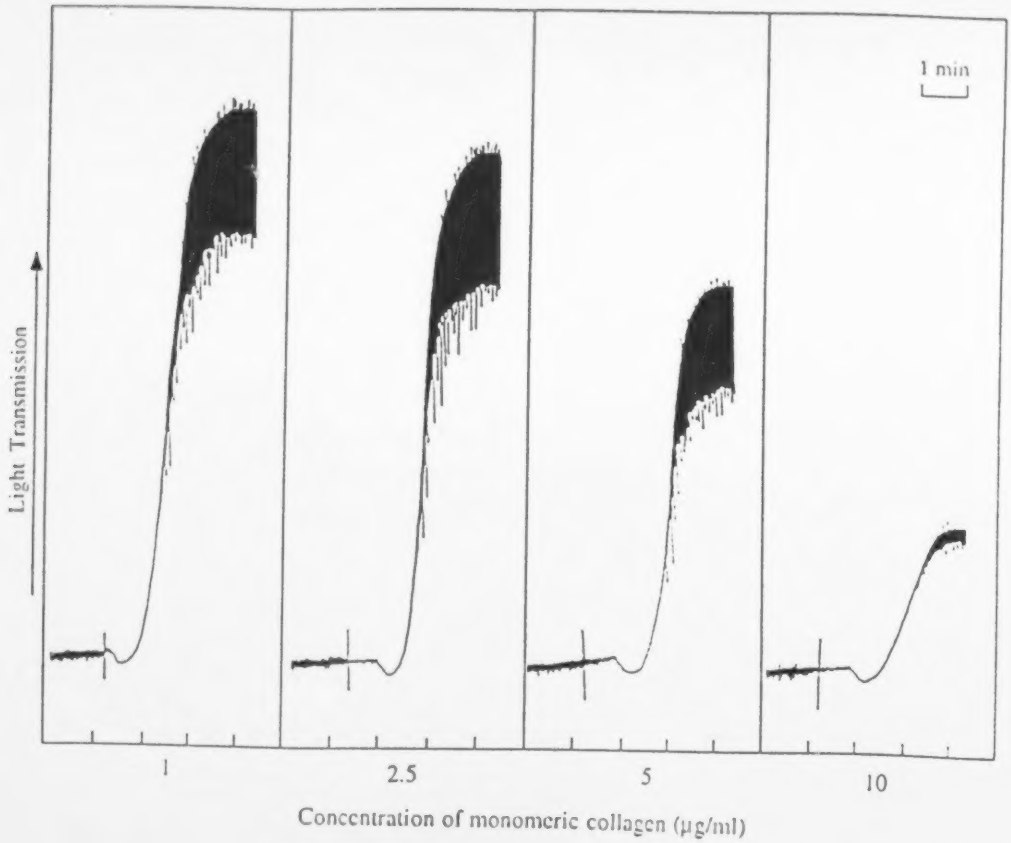


Fig.2: *Collagen bead-induced platelet aggregation: effect of monomeric collagen.* Soluble monomeric collagen was able to inhibit platelet aggregation induced by the same monomeric collagen immobilized on beads in a dose-dependent fashion. Concentrations of soluble monomeric collagen are shown on the bottom of the figure.

Figure 3

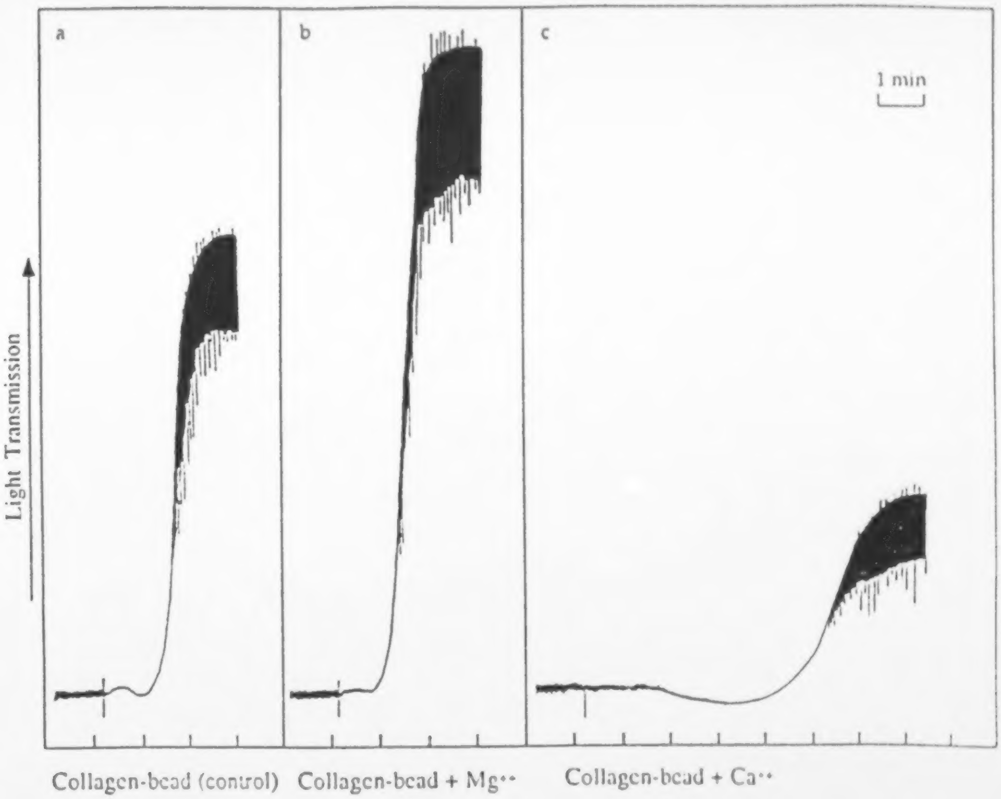


Fig.3: *Collagen bead-induced platelet aggregation: Mg^{2+}-dependency.* Collagen-bead alone does not induce maximal platelet aggregation (a). Aggregation is enhanced in the presence of 2 mM Mg^{2+} (b). Aggregation is inhibited in the presence of 2 mM Ca^{2+} (c).

Figure 4

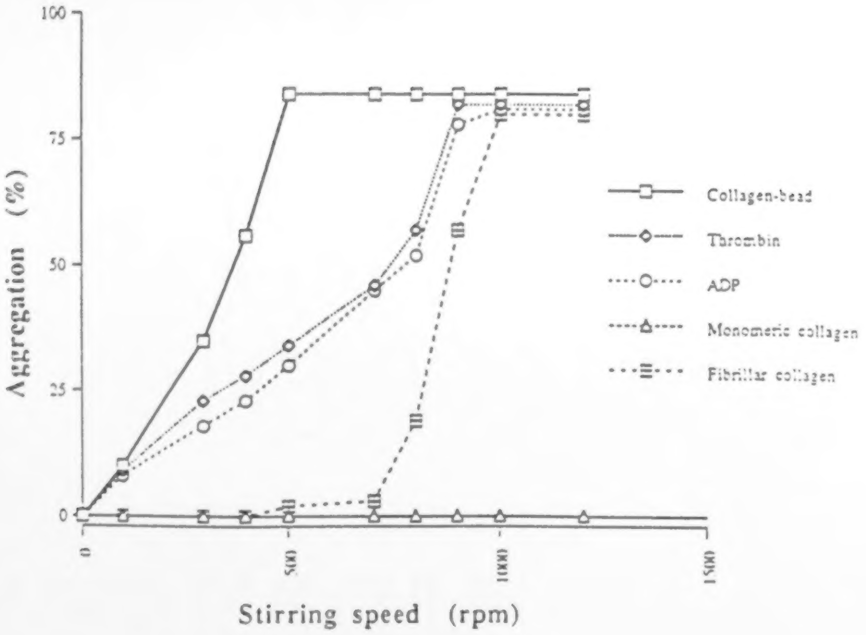


Fig.4: *Platelet aggregation induced by different agonists: relationship to stirring speed.* Platelet aggregation induced by different agonists was evaluated while varying the speed at which the platelet-bead mixture was stirred. Washed platelets were stimulated with either 0.4 µg/ml bead-bound monomeric collagen, thrombin 0.05 U/ml, ADP 20µM, monomeric collagen 10 µg/ml, or fibrillar collagen 1 µg/ml. Percentage of aggregation was calculated as described in Materials and Methods. Each point in this figure indicates the mean of three or more independent determinations on a single sample. Similar results were obtained in at least two additional separate experiments.

Figure 5

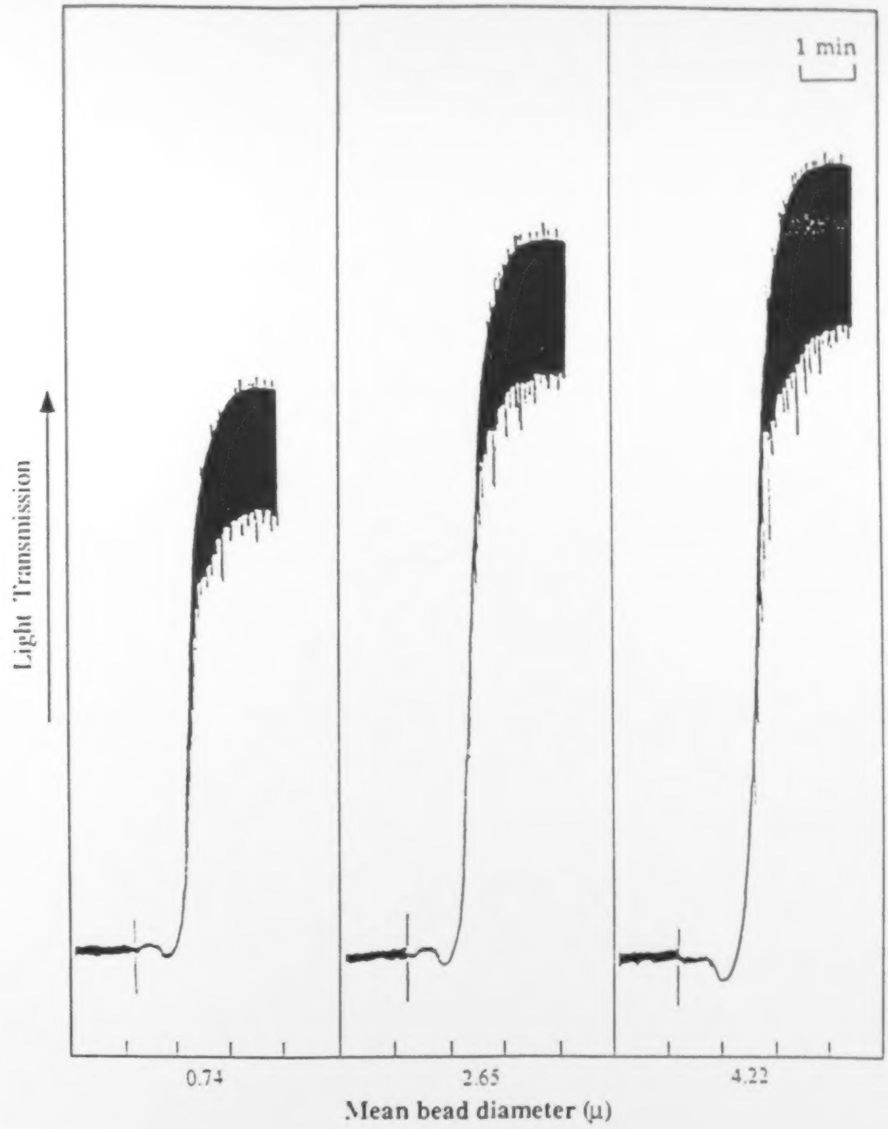


Fig.5: *Collagen bead-induced platelet aggregation: effect of bead-size.* Different sizes of latex beads were used to immobilize monomeric collagen according to Materials and Methods. The larger collagen-bead induced a stronger response (discussed in text).

Figure 6

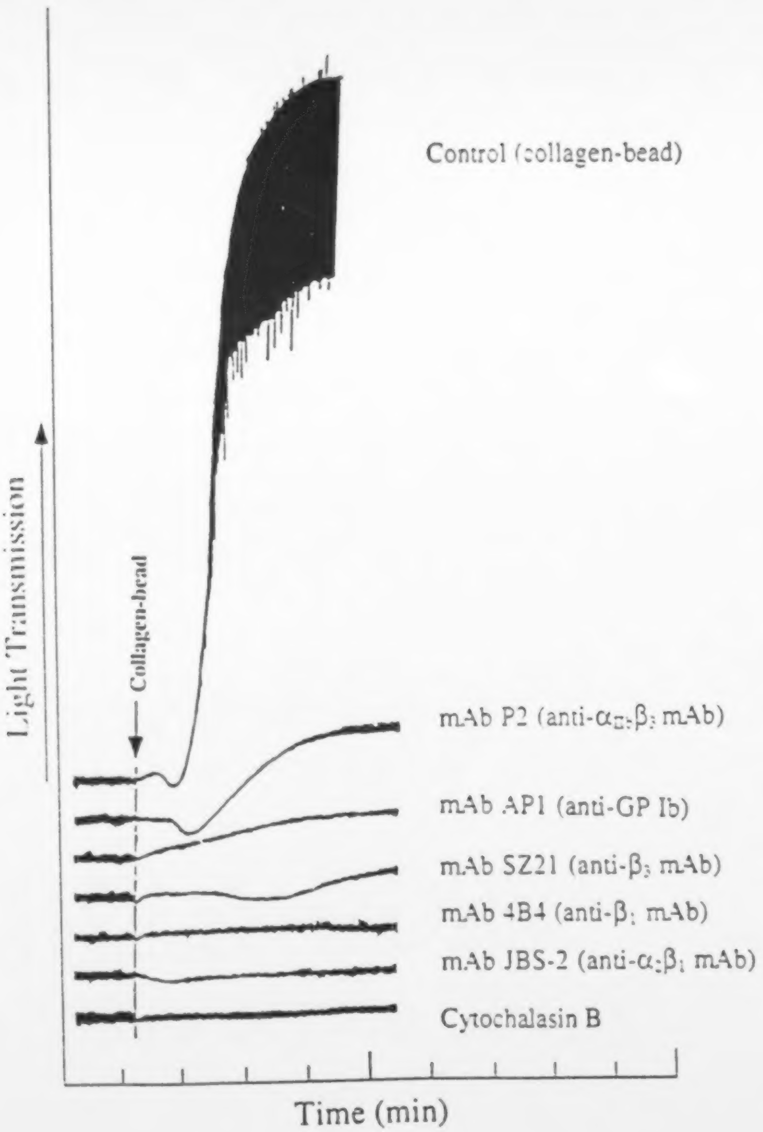


Fig.6: *Collagen bead-induced platelet aggregation: effect of mAbs and cytochalasin B.* Washed platelets were untreated (control), or incubated with different mAbs (mAb P2 10 µg/ml, mAb AP1 8 µg/ml, mAb SZ21 10 µg/ml, mAb 4B4 9 µg/ml, mAb JBS-2 8 µg/ml) or cytochalasin B (20 µM) as described in Materials and Methods prior to stimulation with collagen-beads.

Figure 7

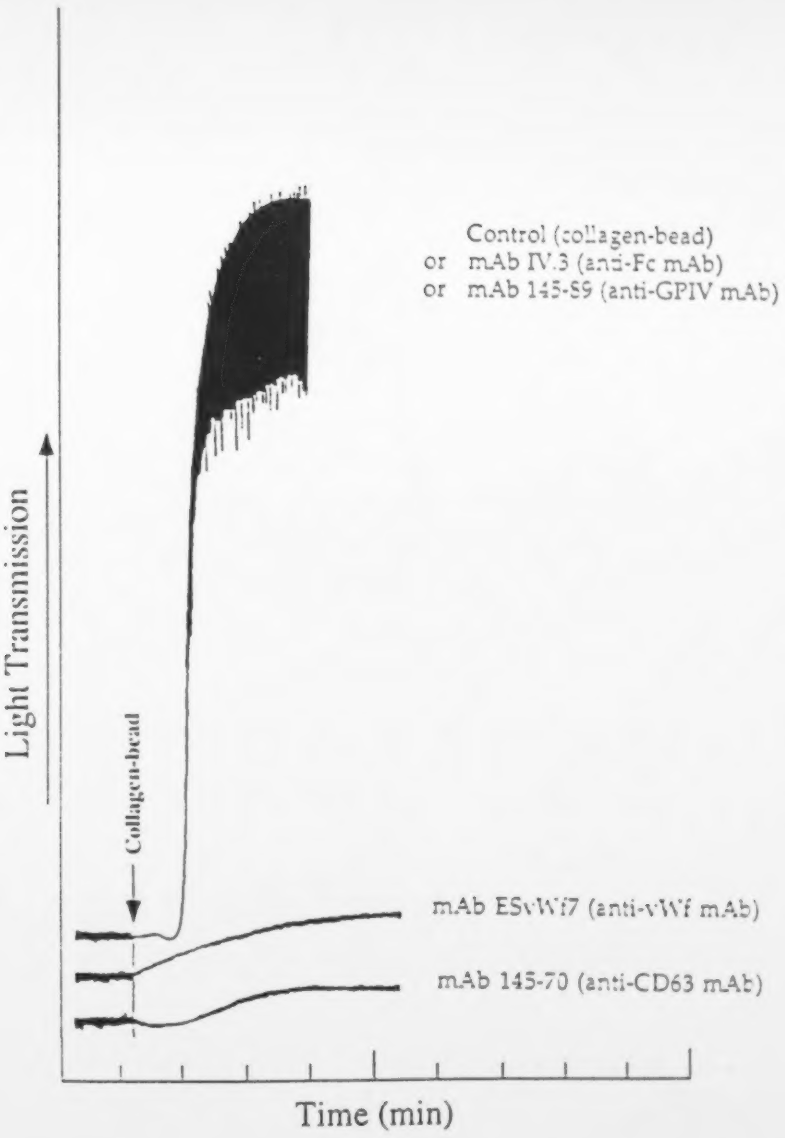
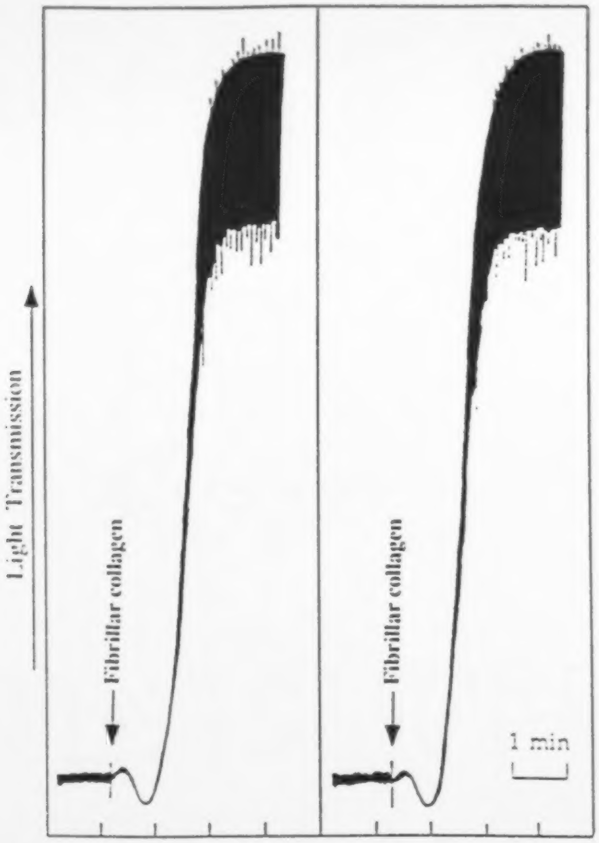


Fig.7: *Collagen bead-induced platelet aggregation: effect of mAbs.* Platelet aggregation was recorded in the absence (control) or presence of mAbs (mAb IV.3 7 μg/ml, mAb 145-89 9 μg/ml, mAb ESvWf7 15 μg/ml, mAb 145-70 6.6 μg/ml) as indicated in Materials and Methods.

Figure 8



Control

mAb 4B4 (anti-β_1 mAb)

Fig.8: *Fibrillar collagen-induced platelet aggregation: effect of anti-β1 mAb 4B4.* Platelet aggregation induced by 1 µg/ml type I fibrillar collagen was recorded prior to incubation of the platelets with 9 µg/ml mAb 4B4 as described in Materials and Methods.

Figure 9

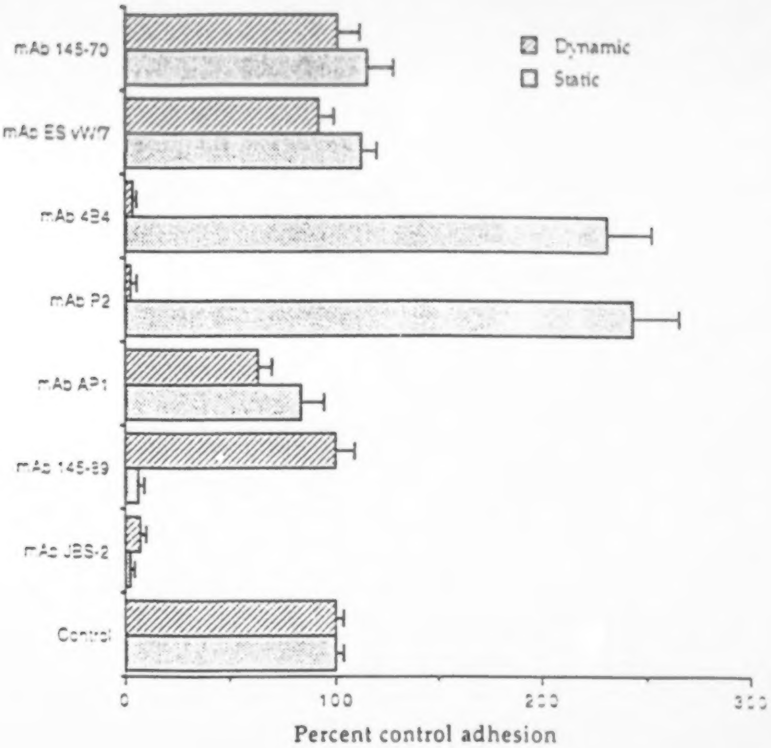


Fig.9: *Comparison of platelet adhesion under dynamic and static conditions: effect of monoclonal antibodies.* The binding of platelets to collagen beads under dynamic conditions (hatched bars) was compared to wells coated with monomeric collagen under static conditions (light shaded bars). Platelets were treated with mAb JBS-2 8 μg/ml, mAb 145-89 9 μg/ml, mAb AP1 8 μg/ml, mAb P2 10 μg/ml, mAb 4B4 9 μg/ml, mAb ESvWf7 15 μg/ml, or mAb 145-70 6.6 μg/ml. Platelet adhesion to collagen-bead, or to collagen matrices without mAbs was used as 100% adhesion. Percent control adhesion in the presence of mAbs was calculated as described in Materials and Methods. The results are the mean of 4 to 8 experiments. Error bars indicate one standard deviation.

Figure 10

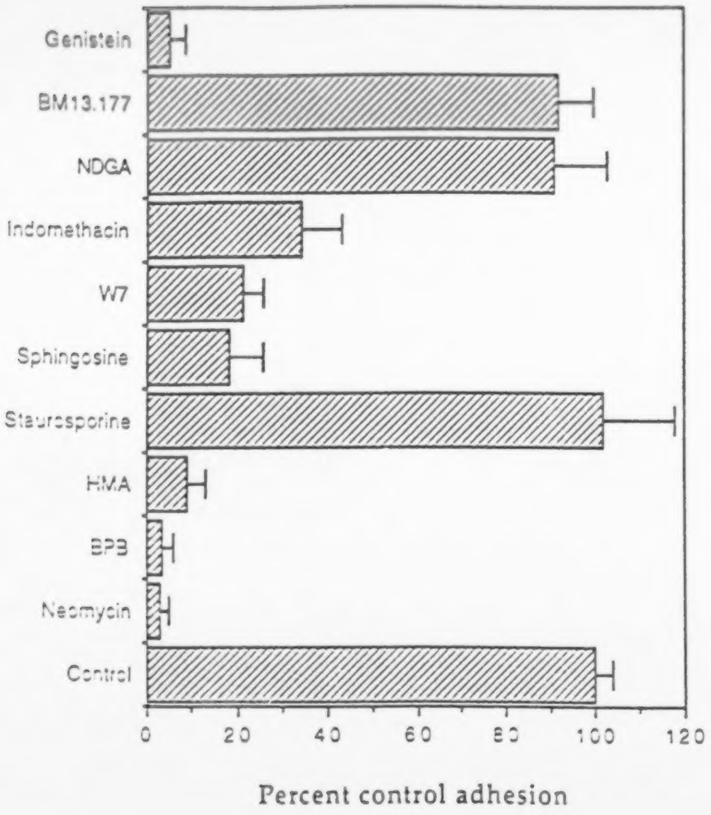


Fig.10: *Platelet adhesion to collagen beads under dynamic conditions: effect of pharmacological inhibitors.* Platelets were treated with neomycin 0.1 mM, BPB 20 μM, HMA 40 μM, staurosporine 0.2 μM, sphingosine 30 μM, W7 50 μM, indomethacin 20 μM, NDGA 3 μM, BM13.177 40 μM, or genistein 15 μg/ml. Platelet adhesion to collage-bead without inhibitors was used as 100% adhesion. Percent control adhesion in the presence of inhibitors was calculated as described in Materials and Methods. The results are the mean of 4 to 8 experiments. Error bars indicate one standard deviation.

Figure 11

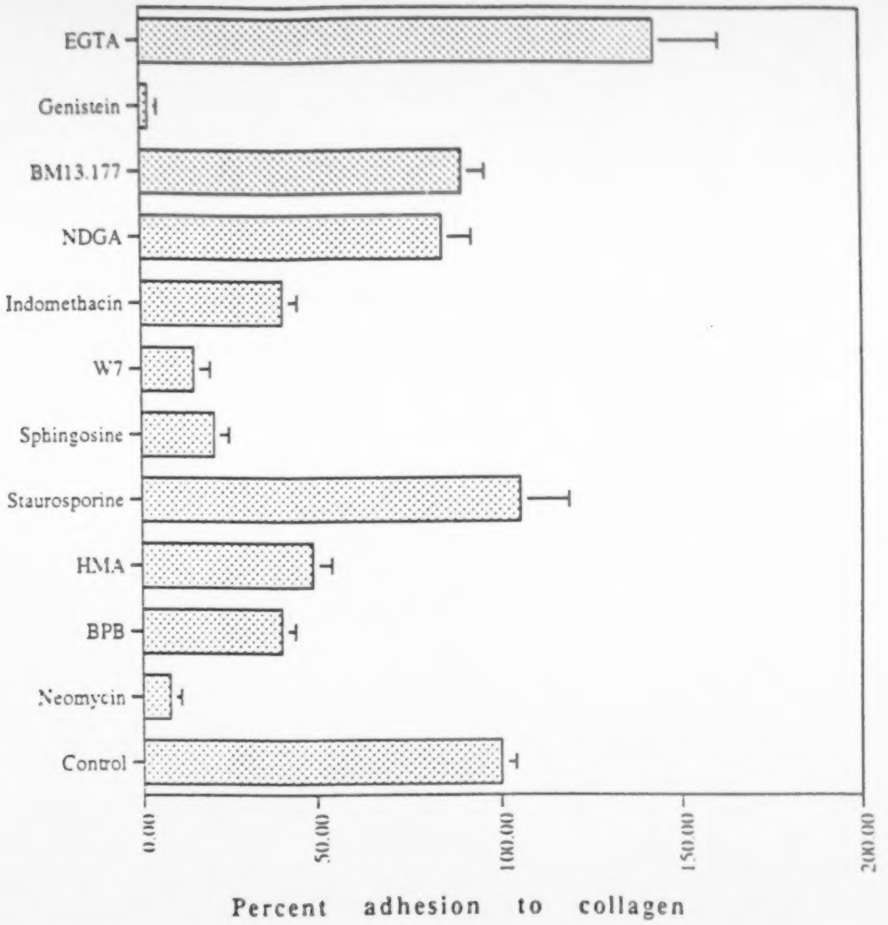


Fig.11: *Platelet adhesion to monomeric collagen under static conditions: effect of pharmacological inhibitors.* The procedure of platelet adhesion to monomeric collagen under static conditions was described in Materials and Methods. Platelets were treated with neomycin 0.1 mM, BPB 20 μM, HMA 40 μM, staurosporine 0.2 μM, sphingosine 30 μM, W7 50 μM, indomethacin 20 μM, NDGA 3 μM, BM13.177 40 μM, genistein 15 μg/ml, or EGTA 4 mM. In the absence of inhibitors platelet binding to collagen was used as 100% adhesion. Percent adhesion to collagen matrices was calculated as described in Materials and Methods. The results are the mean of 4 to 8 experiments. Error bars indicate one standard deviation.

Figure 12

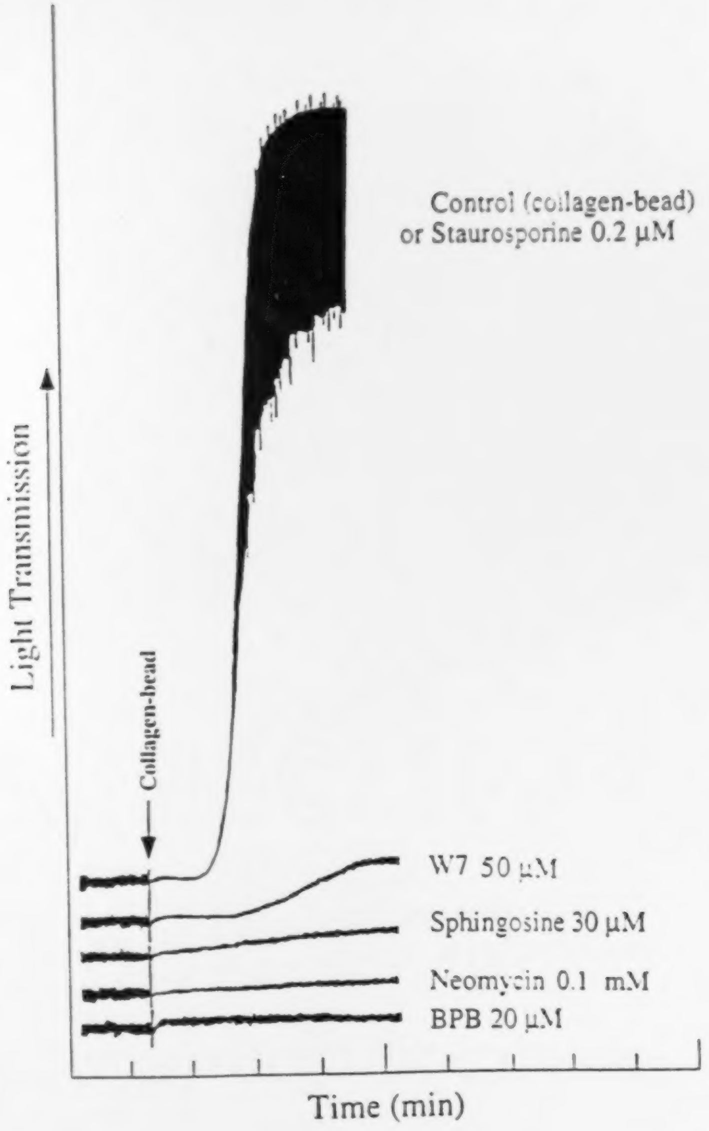


Fig.12: *Collagen bead-induced platelet aggregation: effect of pharmacological inhibitors.* The ability of various pharmacological inhibitors (BPB 20 μM, neomycin 0.1 mM, sphingosine 30 μM, W7 50 μM, or staurosporine 0.2 μM) to inhibit collagen-bead induced platelet aggregation was tested, and the aggregation was recorded. Since staurosporine had no effect on platelet aggregation by collagen-bead, only a control trace was shown.

Figure 13

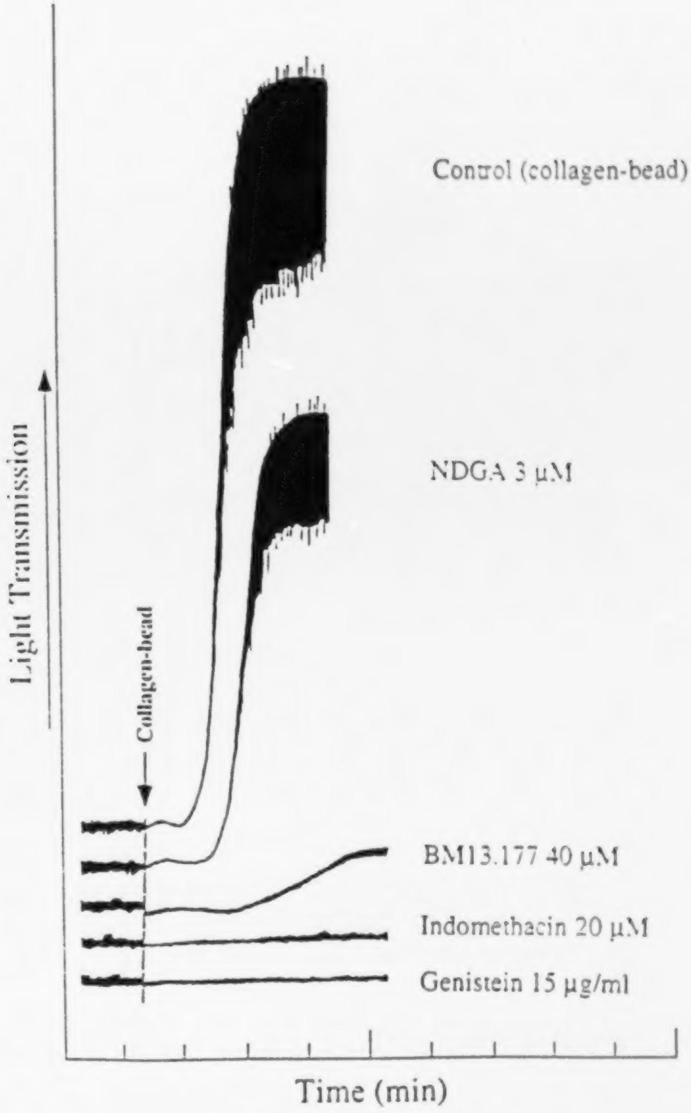


Fig.13: *Collagen bead-induced platelet aggregation: effect of pharmacological inhibitors.* The inhibitory effect of collagen-bead induced platelet aggregation was tested with genistein 15 µg/ml, indomethacin 20 µM, BM13.177 40 µM, or NDGA 3 µM. The experiments was carried out as described in Materials and Methods.

Figure 14

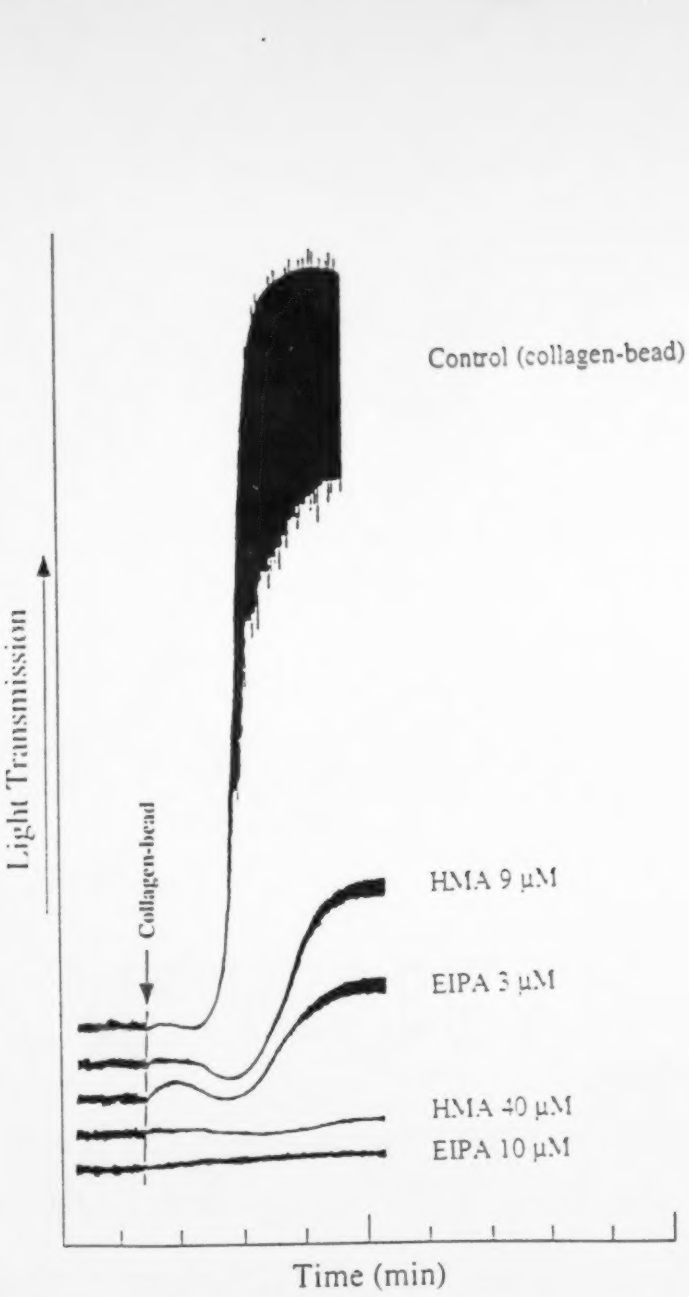


Fig.14: *Collagen bead-induced platelet aggregation: effect of Na^+/H^+ exchange inhibitors.* The experiments were performed as described in Materials and Methods. The ability of Na^+/H^+ exchange blockers (EIPA 3 µM or 10 µM, and HMA 9 µM or 40 µM) to inhibit collagen-bead induced platelet aggregation was tested.

Figure 15

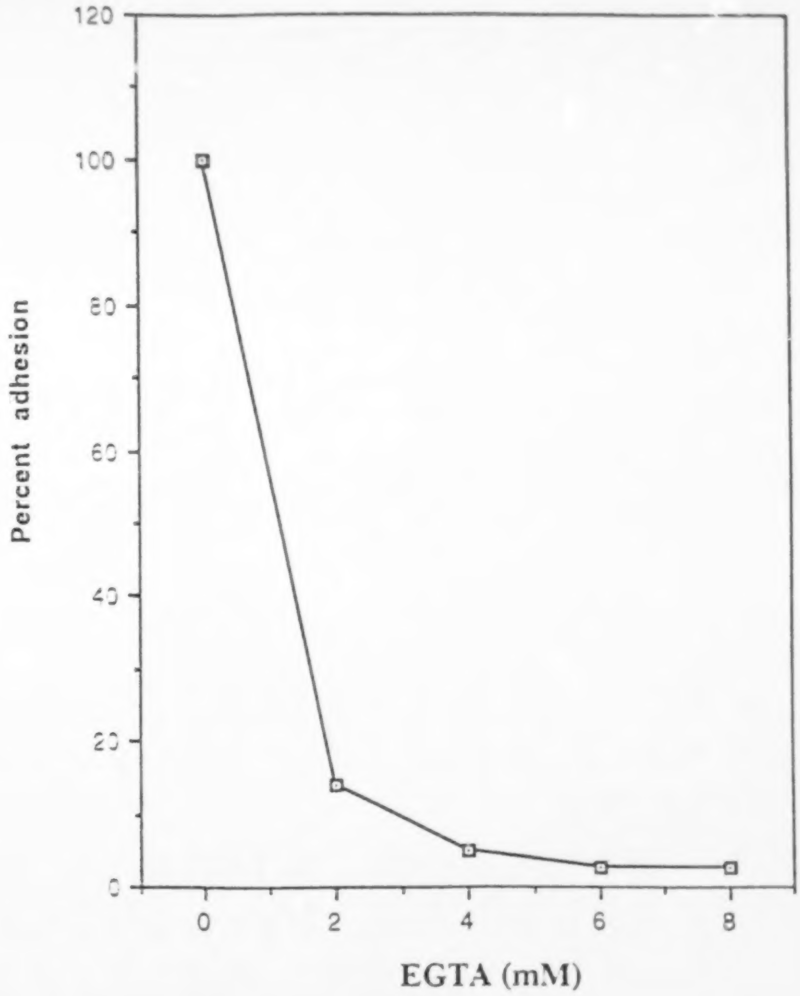


Fig.15: *Platelet adhesion to collagen beads under dynamic conditions: effect of EGTA.* Platelets were preincubated with varying concentrations of EGTA for 1 minute prior to stimulation with collagen beads. The number of platelet adhesion to collagen beads in the absence of EGTA was considered as 100% binding. Percent adhesion to the beads was calculated as described in Materials and Methods. Each point in this figure indicates the mean of at least three independent determinations on a single sample. Similar results were obtained in three additional separate experiments.

Figure 16

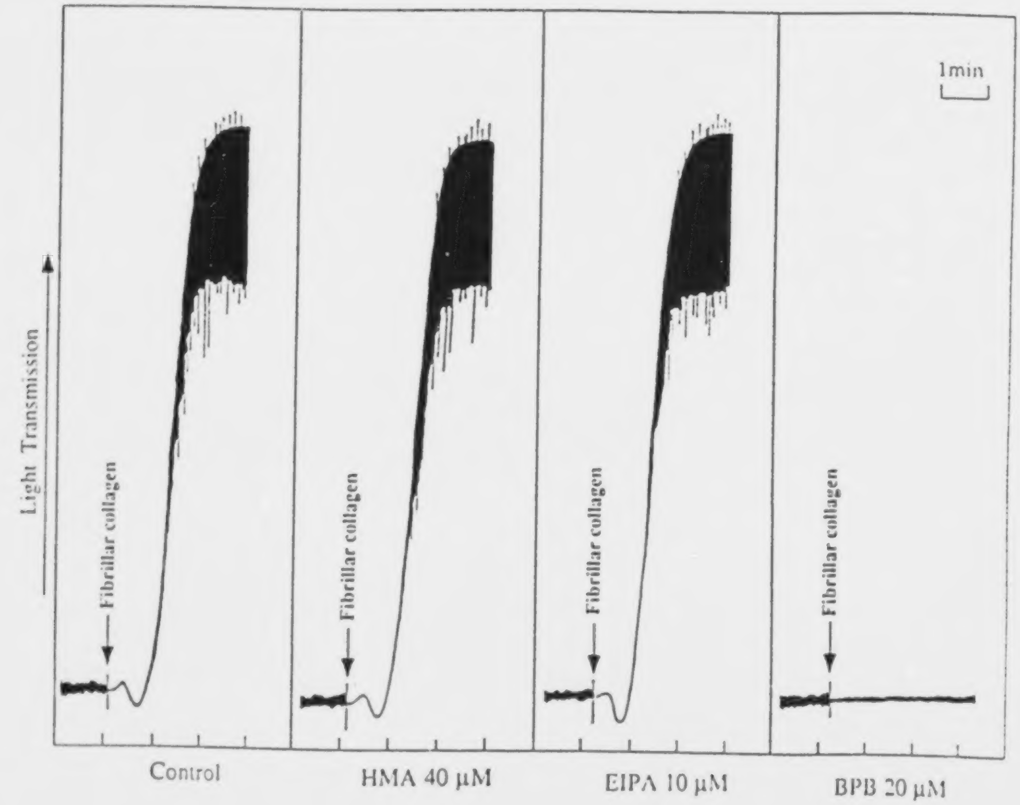


Fig.16: *Fibrillar collagen-induced platelet aggregation: effect of pharmacological inhibitors.* Platelet aggregation induced by 1 μg/ml fibrillar collagen was inhibited by preincubation of the platelets with 20 μM BPB. Na^+/H^+ exchange blockers HMA (40 μM) or EIPA (10 μM) had no effect on fibrillar collagen-induced platelet aggregation.

Figure 17

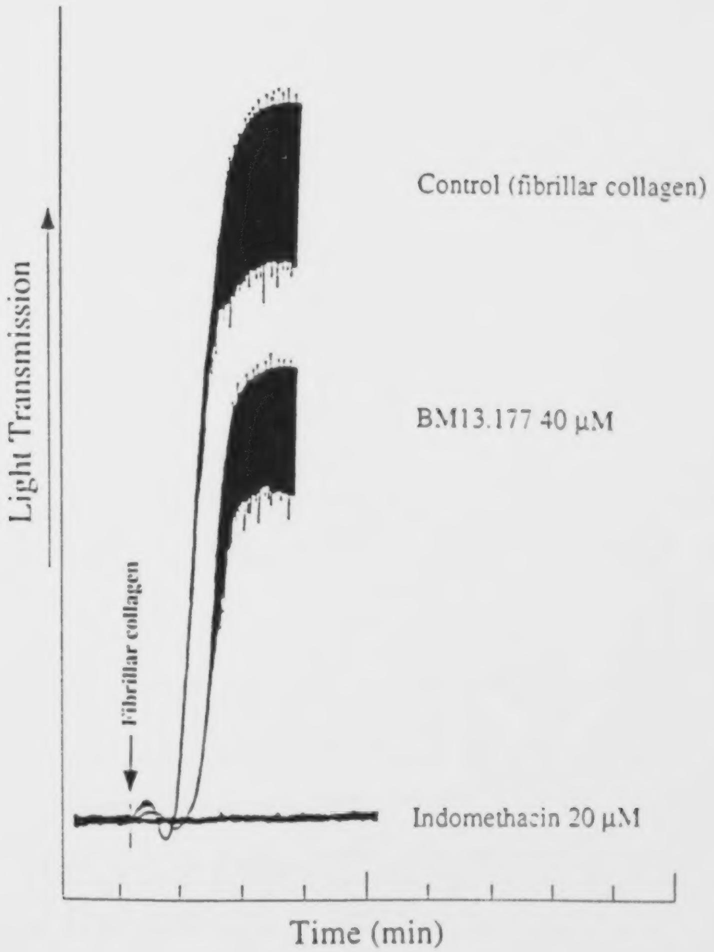


Fig.17: *Fibrillar collagen-induced platelet aggregation: effect of pharmacological inhibitors*. The aggregation assay was performed as described in Materials and Methods. Fibrillar collagen (1 µg/ml) induced platelet aggregation was completely blocked by 20 µM indomethacin, and partially blocked by 40 µM BM13.177.

Figure 18

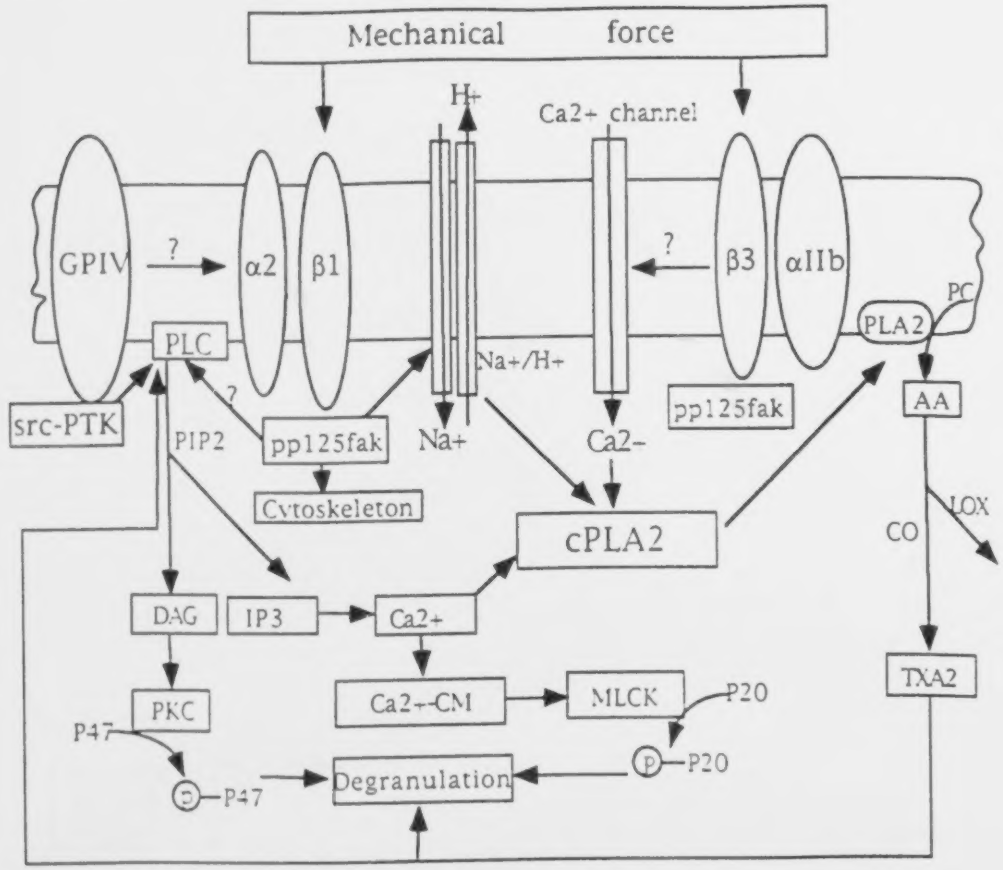

Mechanical force
H+
Ca2+ channel
GPIV
?
α2
β1
?
β3
αIIb
PC
PLA2
PLC
Na+/H+
src-PTK
?
PIP2
pp125fak
Na+
Ca2-
pp125fak
AA
Cytoskeleton
cPLA2
CO
LOX
DAG
IP3
Ca2+
TXA2
PKC
Ca2+-CM
MLCK
P20
P47
P—P20
P—P47
Degranulation

Fig.18: *Model of platelet activation by collagen.* Platelet activation by collagen is mainly mediated by β1 integrin which may be coupled with Na^+/H^+ exchange, and by β3 integrin which may be related to importation of Ca^{2+} through stress-operated Ca^{2+} channel. The activation of Na^+/H^+ exchange and importation of Ca^{2+} in turn stimulate cytosolic PLA_2 ($cPLA_2$) which results in a series of intracellular responses. Mechanical force was suggested to be involved in the process of collagen-mediated platelet activation through β1 and β3 integrins.

Abbreviation: GP, glycoprotein; PLC, phospholipase C; PLA2, phospholipase A2; PC, phosphatidylcholine; Ca^{2+}-CM, calcium-calmodulin; PIP2, phosphatidylinositol; AA, arachidonic acid; CO, cyclooxygenase; TXA2, thromboxane A2; PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol trisphosphate; LOX, lipoxygenase; p47, IP3 phosphomonoesterase; MLCK, myosin light chain kinase; p20, myosin light chain; cPLA2, cytosolic PLA2; src-PTK, src-related protein tyrosine kinase; pp125fak, focal adhesion kinase.

Table 1*

Platelet membrane glycoproteins

RECEPTOR	LIGAND
Adhesion	
Integrin*	
GPIa/IIa (VLA-2)	Collagen
GPIc/IIa (VLA-6)	Laminin
GPIc☆/IIa (VLA-5)	Fibronectin
A_v/IIIa	Vitronectin, fibrinogen, von Willebrand factor. thrombospondin
GPIIb/IIIa	Fibrinogen, fibronectin, von Willebrand factor. vitronectin (? thrombospondin)
Others	
GPIb	von Willebrand factor
GPIV	Thrombospondin, collagen
Aggregation	
GPIIb/IIIa	Fibrinogen, fibronectin, von Willebrand factor. vitronectin (? thrombospondin)

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* from Coller New Eng. J. Med. 322:33, 1990

Table 2

List of monoclonal antibodies and their function

Monoclonal antibody (mAb)	Recognized protein	
Anti-integrin mAbs		
mAb 4B4	GPIIa	(β1)
mAb LYP22	GPIIa	(β1)
mAb JBS-2	GPIa/IIa	(α2β1)
mAb P1H5	GPIa/IIa	(α2β1)
mAb 12F1	GPIa/IIa	(α2β1)
mAb SZ21	GPIIIa	(β3)
mAb P2	GPIIb/IIIa	(αIIbβ3)
mAb P256	GPIIb/IIIa	(αIIbβ3)
Other mAbs		
mAb AP1	GP Ib	
mAb SZ 2	GPIb	
mAb AN51	GPIb	
mAb 145-89	GPIV (CD36)	
mAb 145-70	CD63	
mAb IV.3	Fc receptor	
mAb ESvWf7	vWf	